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Dark septate root endophytic (DSE) fungi in Canada: Geographic distribution and patterns of variation

by

Melissa M. Piercey

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Dark septate root endophytic (DSE) fungi in Canada: Geographic distribution and patterns of variation** submitted by Melissa M. Piercey in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Dark septate root endophytes (DSE) are an artificial assemblage of fungi that have darkly pigmented, septate hyphae and that are frequent or distinctive intracellular associates of roots of apparently healthy plants. The distribution of DSE fungi was examined along a latitudinal transect in Canada running from the high arctic to the 49th parallel. DSE fungi were isolated from all latitudes, but the species isolated from roots changed with latitude. Patterns of genetic variation across the latitudinal transect of *Phialocephala fortinii*, a common DSE fungus, were also examined through analysis of amplified fragment length polymorphisms (AFLP). No clones were detected, and neighbour-joining analysis of genetic distances yielded eight well-supported clusters, four of which consisted of individuals from more than one latitude. Some population subdivision was detected, with the majority of genetic variation attributable to differences between individuals within each site. Some linkage disequilibrium was detected, which may be in part a consequence of partial clonality.

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Chapter 1: Overview of the dark septate root endophytic fungi

1.1 Dark septate root endophytes: definition and history

Dark septate root endophytes (DSE) are an artificial assemblage of fungi that have darkly pigmented, septate hyphae and are frequent or distinctive intracellular associates of roots of apparently healthy plants. This definition does not exclude taxa isolated from soil or non-living organic material, provided the identity of these fungi have been linked to taxa known to form intracellular associations in roots. The DSE fungi are ubiquitous in the roots of plants in most cold- and nutrient-stressed habitats (Richard & Fortin 1974) and are circumboreal in distribution (Jumpponen & Trappe 1998a).

Melin (1922) was the first to describe DSE fungi when he reported a sterile grey fungal associate of the roots of *Pinus sylvestris* and *Picea abies* from 40 different localities in Europe, and named it *Mycelium radicis atrovirens* (MRA). These fungi did not form typical ectomycorrhizas, with a well-defined mantle and Hartig net, but rather grew exclusively intracellularly and formed microsclerotia (assemblages of deeply melanized, compact irregular cells; Stoyke & Currah 1991) within root cells (Melin 1922). This distinctive morphological association with plant roots was considered "pseudomycorrhizal" (Melin 1922). A typical pseudomycorrhiza has, in addition to microsclerotia, hyphae that penetrate the outer cortical and epidermal cells of a root, with hyphal growth parallel to the main axis of the root between and through cells (Jumpponen & Trappe 1998a). The term MRA has since become synonymous with the term dark septate endophyte (DSE; Haselwandter & Read 1982; Jumpponen & Trappe 1998a).

In spite of their superficial similarities, it is impossible to make assumptions about the common role of different species of DSE fungi in ecosystems because the nature of their associations with plants varies widely. In resynthesis studies, some strains of DSE will form ectomycorrhizas, with a Hartig net and discontinuous mantle (Fernando & Currah 1996), or ectendomycorrhizas, with a thin mantle and Hartig net combined with hyphal penetration into root epidermal and cortical cells (Wilcox & Wang 1987, Yu Egger & Peterson 2001). In some cases, DSE have been observed to form mutualistic relationships by increasing the dry weight of host plants (e.g. Wang & Wilcox 1985, Fernando & Currah 1996), in others cases parasitic relationships by decreasing the dry weight or increasing mortality of hosts (e.g. Melin 1922, Wilcox & Wang 1987, Stoyke *et al.* 1992), and in still others, no effects are apparent (e.g. Stoyke & Currah 1991, O'Dell *et al.* 1993).

DSE fungi are known from the roots of over 600 species of plants representing 114 different families (Jumpponen & Trappe 1998a), and from soil (e.g. Gams 1963, Ahlich *et al.* 1998, Grünig *et al.* 2001) and rotting wood (e.g. Kendrick 1961, Lumley *et al.* 2001). The DSE assemblage is polyphyletic (LoBuglio *et al.* 1996, Jumpponen & Trappe 1998a), consisting of ascomycetous fungi linked only by the dark pigmentation of their hyphae and their frequent isolation from the roots of apparently healthy plants. Many DSE fungi have not sporulated in culture and their identity remains unknown. These fungi are frequently referred to in the literature by arbitrary strain identifiers, although in some cases their phylogenetic placement has been determined by molecular methods (e.g. "Type 1" closely related to *Phialocephala fortinii*, Grünig *et al.* 2002; "Unknowns 1 and 2" allied with the Pleosporales, "Unknowns 3 and 4" allied with either the Leotiales or the Pezizales, Jumponnen & Trappe 1998a). Without names, these non-taxonomic designations become confusing.

Presented below are detailed descriptions of several species of DSE fungi, illustrating the morphological and ecological diversity within this group.

1.2 Representative species of DSE fungi

Of the DSE fungi that have sporulated in culture, five hyphomycetous taxa have been named: *Phialocephala dimorphospora* Kendrick, *Phialophora finlandia* Wang & Wilcox, *Chloridium paucisporum* Wang & Wilcox, *Phialocephala fortinii* Wang & Wilcox, and *Leptodontidium orchidicola* Sigler & Currah.

1.2.1 *Phialocephala dimorphospora*

The original description of *P. dimorphospora* by Kendrick (1961) was based on an isolate from rotten deciduous wood. Subsequently, 15 strains of *P. dimorphospora* were identified among cultures of *Mycelium radicis atrovirens* from herbaria (living cultures were obtained, but herbaria were not named) and isolated from short rootlets of *Picea mariana* (Richard & Fortin 1974). Colonies of *Phialocephala dimorphospora* on potato dextrose agar are dark mouse grey with a black reverse. Hyphae are brown with smooth to very rough walls (Kendrick 1961). The sporogenous apparatus of *P. dimorphospora* is a darkly pigmented, complex obconical sporogenous head 30-70 µm in length and has 2-5 series of metulae bearing a cluster of phialides, borne at the apex of a stipe, 19-81 µm long (Kendrick 1961). Phialides are 10-8-17.5 X 2.5-3.1 µm, hyaline, subcylindrical and have a long cylindrical collarette. Primary (first formed) conidia are ovoid, 3.6-5.4 X 2.3-2.8 µm, and secondary or subsequent conidia are spherical and 1.7-2.4 µm in diam. The conidia accumulate in a slimy head at the apex of the sporogenous apparatus (Kendrick 1961). *Phialocephala dimorphospora* has been isolated from soil, rotting wood and roots, and has been reported from soil and plant roots in both North America and Europe (Table 1.1).

1.2.2 *Chloridium paucisporum*

Wang & Wilcox (1985) were able to induce sporulation in, and subsequently name, three species of hyphomycetes (*Chloridium paucisporum*, *Phialophora finlandia* and *Phialocephala fortinii)* from a collection of DSE fungi isolated from the roots of conifers. The least-studied of these is *Chloridium paucisporum*, which was isolated only once from *Pinus resinosa*, where it formed ectendomycorrhizas characterized by intracellular chlamydospore-like structures, a thin black mantle 3-12 µm thick, and a fine-textured Hartig net (Wilcox & Ganmore-Neumann, 1974, Wilcox *et al*. 1974). This species has also been grown in culture with *Pinus resinosa*, *Picea rubens* and *Betula alleghaniensis* to produce both ectomycorrhizas (black mantle and Hartig net) and ectendomycorrhizas (both inter- and intracellular colonization of root cortex), with miscosclerotia in inner cortical cells (Wilcox & Wang 1987). *Chloridium paucisporum* is characterised by dark grey colonies with white margins, smooth dematiaceous hyphae, solitary or branched, darkly pigmented conidiophores, 20-70 µm long, with long, thin phialides (10-25 µm X 1.5-2.5 µm) that are darkly pigmented at the base and hyaline at the apex, which terminates in a conspicuous hyaline collarette (Wilcox *et al.* 1974, Wang & Wilcox 1985). Conidia are hyaline, smooth and single-celled, 3-5 µm X 1.5 µm and vary in shape from cylindrical to ovoid or reniform (Wilcox *et al.* 1974, Wang & Wilcox 1985). The phylogenetic position of *C. paucisporum* is unknown.

1.2.3 *Phialophora finlandia*

Phialophora finlandia is also reported infrequently in the literature. Originally isolated from the roots of a *Pinus sylvestris* seedling (Wang & Wilcox 1985) in Finland, it has since been reported only from the root tip of a *Pinus strobus* seedling in Canada (Schelkle *et al.* 1996) and from the root of *Arctostaphylos uva-ursi* (Hambleton, pers. comm.) This hyphomycete is characterised by dark grey, floccose colonies on Modified Melin Norkran's agar (MMN) with smooth to verrucose dematiaceous hyphae (Wang & Wilcox 1985). Conidiophores are brown, smooth-walled and many-branched; conidiogenous cells are phialidic, light brown, with conspicuous pale brown collarettes. The conidiogenous cells, including collarette, are (15)18-20(-29) µm X (2-)2.5(-3) µm. Conidia are 4.5-6 X 1.5-2 µm, single-celled, ellipsoidal, smooth-walled, subhyaline to light brown. *Phialophora finlandia* forms ectendomycorrhizal associations with conifers (Wang & Wilcox 1985; Wilcox & Wang 1987) and is relatively close phylogenetically to the *Hymenoscyphus ericae* – "*Piceirhiza bicolorata*" ectomycorrhizal morphotype aggregate based on internal transcribed spacer (ITS) and small subunit ribosomal DNA (5.8S) sequence analysis (Vrålstad *et al.* 2002). Although its teleomorph is unknown, analysis of DNA sequence data indicates that *P. finlandia* is ascomycetous and belongs in the Leotiales (Vrålstad *et al.* 2002).

1.2.4 *Phialocephala fortinii*

Of the DSE fungi listed above, *Phialocephala fortinii* has been studied the most intensively because it is easily isolated and grown in culture, and has distinctive morphological features. *Phialocephala fortinii* was first described as a pseudomycorrhizal associate of *Pinus sylvestris* (Wang & Wilcox 1985) and has since been isolated from the roots of 52 plant species representing 18 families (Table 1.1). Cultural characters in *P. fortinii* vary widely. Colonies may have abundant aerial mycelium, or little aerial mycelium but abundant submerged mycelium, numerous sclerotia or none at all, and solid or feathery margins (Stoyke 1991, Addy *et al.* 2000). All isolates have wart-like blisters on aerial hyphae and, when sporulating, produce dense cone-shaped clusters of conidiogenous cells borne at the apex of conidiophores of variable length (10-120 µm) (Wang & Wilcox 1985, Currah & Tsuneda 1993). Phialides, with collarettes and up to 15µm in length, are brown and produced apically on a complex obconical sporogenous head consisting of up to seven series of divergent compact metulae (Wang & Wilcox 1985). Conidia are minute, hyaline, and single-celled; primary conidia are obvoid, 3 X 1-1.5 µm, and secondary conidia are globose to subglobose, 1.5-2 µm diam. (Wang & Wilcox 1985). *Phialocephala fortinii* is similar to *P. dimorphospora* but that species is distinguished on the basis of its smooth conidiophores, smaller and less complex conidiogenous heads, darker collarettes, and larger conidia (Wang & Wilcox 1985).

In roots, *Phialocephala fortinii* typically produces pseudomycorrhizas, typified by microsclerotia and intracellular penetration of root cells. Ectomycorrhiza-like structures, with Hartig net and patchy mantle, were also observed in one instance (Fernando & Currah 1996). Detailed studies of the ingress of *P. fortinii* into the roots of *Rhododendron brachycarpum* (Ericaceae) (Currah *et al.* 1993) and *Asparagus officinalis* (Asparagaceae) (Yu Nassuth & Peterson 2001) provide a more complete picture of how this species interacts with host roots. *Phialocephala fortinii* grew over the root tips of young *R. brachycarpum* seedlings, inhibiting differentiation and elongation (Currah *et al.* 1993); this was also observed by Stoyke & Currah (1993) with another ericaceous host, *Menziesia ferruginea*. When older *R. brachycarpum* and *A. officinalis* seedlings were inoculated, hyphae grew along the root surface (Currah *et al.* 1993; Yu Nassuth & Peterson 2001). First a loose network of hyphae formed on the root surface, followed by penetration into epidermal cells by swollen appressorium-like structures (Yu Nassuth & Peterson 2001). From here hyphae penetrated the exodermal and cortical cells, and in some cases also colonized vascular tissue (Currah *et al.* 1993, Yu Nassuth & Peterson 2001). Microsclerotia formed within the epidermal and exodermal cells as well as outside the root itself (O'Dell *et al.* 1993, Currah *et al.* 1993) and accumulated glycogen, protein and phosphorus (Yu Nassuth & Peterson 2001).

Strains of *Phialocephala fortinii* positively affected biomass and nutrient acquisition by *Pinus contorta* in axenic culture on MMN of varying glucose concentrations, but these same strains did not produce any noticeable effects on *Pinus contorta* in open pot cultures (Jumpponen & Trappe 1998b). It appears that this species forms a non-specific association with its host plant, having the ability to play a number of roles depending on substrate conditions, identity of host, and strain of *P. fortinii*, as exemplified in a variety of responses observed in at least seven independent studies: *Phialocephala fortinii* stunted the growth *Pinus resinosa* and *Picea rubens* in axenic culture on modified Melin-Norkrans agar (MMN) with the negative effects increasing with pH of the growth medium (Wilcox & Wang 1987). It increased the mortality of *Menziesia ferruginosa* grown axenically on cellulose agar (Stoyke & Currah 1993), but increased the shoot dry weight of *Potentilla fruticosa* in open pot cultures (Fernando & Currah 1996). The presence of *P. fortinii* did not noticeably affect the growth rate of *Menziesia ferruginea* in axenic culture on cereal agar (Stoyke & Currah 1991), *Rhododendron brachycarpum* in open pot cultures (Currah *et al.* 1993), *Pinus contorta* and *Lupinus latifolia* in growth pouches fertilized with half-strength Melin-Norkrans nutrient solution (O'Dell *et al.* 1993), and *Picea glauca* in open pot cultures (Fernando & Currah 1996). Protection against verticillium wilt in *Solanum melongena* seedlings was also conferred when seedlings were pre-inoculated with *P. fortinii* (Narisawa *et al.* 2002).

1.2.5 *Leptodontidium orchidicola*

Leptodontidium orchidicola was first isolated from the root of an orchid, *Platanthera huronensis* (Linnaeus) Lindley (Currah *et al.* 1987) and since has been isolated from the roots of 17 other plant species (Jumpponen & Trappe 1998a) including other orchids (e.g. *Calypso bulbosa*, Currah *et al.* 1988), herbaceous plants (e.g. *Carex* sp., Fernando & Currah 1995) and woody plants (e.g. *Rubus* sp. and *Abies balsamea*, Fernando & Currah 1996). *Leptodontidium orchidicola* has been isolated from sites in Canada and Japan, but it is conceivable that it may be misidentified among collections of *P. fortinii* made elsewhere, because the two species are remarkably similar. *Leptodontidium orchidicola* is characterized by a grey colony on corn meal agar (CMA). Isolates produce single-celled blastic conidia either along both lateral and terminal walls of hyaline or pigmented conidiogenous hyphae, or on swollen or unswollen conidiogenous cells produced on the same hypha (Fernando & Currah 1995). Its teleomorph is unknown, but affinity is among the ascomycetes, based on observations of septal ultrastructure (simple septum with Woronin bodies; Currah & Sherburne 1992) and through analysis of rRNA sequence data (Jumpponen & Trappe 1998a). Its position among the orders of the Ascomycota is still in question.

1.3 Newly recognized DSE species

In addition to these five species, many other darkly pigmented hyphomycetes with septate hyphae have been isolated from the roots of plants and fit the criteria for inclusion in the DSE, i.e., they have darkly pigmented septate hyphae and are frequent or distinctive intracellular associates of roots of apparently healthy plants. Adhering to these criteria increases the number of fungi recognized as DSE; four of these are included below, selected for their economic importance or particular interest.

1.3.1 *Heteroconium chaetospora*

Heteroconium chaetospira (Grove) Ellis is an olivaceous-brown hyphomycete producing pale brown, fusiform, 0-4 septate conidia (20-35 X 3-4 µm) in acropetal chains on little-differentiated conidiophores (Domsch et al. 1980). Although it has been isolated from rotten wood (Domsch et al. 1980), soil (CBS, Table 1.1) and millipede droppings (Ellis 1976), more relevant here is its isolation from roots of *Ammophila arenaria* (IMI, Table 1.1), *Picea abies* and *Pseudotsuga menziesii* (CBS, Table 1.1). When colonizing the roots of *Solanum melongena* (eggplant), *H. chaetospira* hyphae grow between and within cortical cells, which confers some resistance to verticillium wilt (Narisawa *et al.* 2002).

1.3.2 *Hymenoscyphus ericae*

Hymenoscyphus ericae (Read) Korf & Kernan is the best-known mycorrhizal associate of ericaceous plants, forming typical ericoid mycorrhizas characterized by the presence of coiled hyphae within the individual cortical cells of fine hair roots and the absence of fungal structures external to the root (Read 1996). It can also colonize the roots of *Picea mariana in vitro* (Piercey *et al.* 2002). Strains of *H. ericae* are linked to the *Piceirhiza bicolorata* ectomycorrhizal morphotype (Vrålstad *et al.* 2000), a group to which *Phialophora finlandia* is also allied, based on analysis of ITS and small subunit ribosomal DNA sequence data (Vrålstad *et al.* 2002).

Hymenoscyphus ericae is an inoperculate discomycete placed in Helotiaceae (Leotiales), and produces short stipitate, cup-shaped apothecia, up to 1 mm in diam, that are yellowish to orange and bear a dense rim of minute hairs along the margin when mature (Hambleton *et al.* 1999). Asci are 63-77 X 8-10 µm and contain eight ascospores, 6.2-8.8 X 2.3-3.2 µm. In culture, *H. ericae* produces light or dark grey-brown colonies with white margins, flat radiating furrows and a fine nap of aerial hyphae (Egger & Sigler 1993). The anamorph of *H. ericae* is *Scytalidium vaccinii* Dalpé, Litten & Sigler (Egger & Sigler 1993). *Scytalidium vaccinii* produces 0-1 septate cylindrical, hyaline to subhyaline arthroconidia, 3-11 X 1.5-2.5 µm, that remain connected in zigzag chains until dehiscence (Egger & Sigler 1993).

1.3.3 *Oidiodendron maius*

Oidiodendron maius Barron is another well-known ericoid mycorrhizal associate of ericaceous plants (Douglas *et al.* 1989; Hambleton & Currah 1997; Currah *et al.* 1999; Thormann *et al.* 1999). It has also been isolated from forest soil (Barron 1962), decaying wood (Lumley *et al.* 2001), and peat (Thormann *et al.* 2001). This fungus possesses a wide range of enzymatic abilities (Rice & Currah 2002) and is able to degrade peat. This hyphomycete is easily recognizable by its long (up to 350 µm) dematiaceous conidiophore that is tree-like (dendritic) with a spreading crown of arthroconidial branches. Conidia are 3-5 X 2-2.5 µm, subglobose, and broadly ellipsoidal or cylindrical (Barron 1962). *Oidiodendron maius* is a member of the Leotiales, allied with the Myxotrichaceae by sequence analysis of rDNA (Hambleton *et al.* 1998) and by the production of sterile gymnothecia that are morphologically similar to *Myxotrichum arcticum* (Rice & Currah, in press).

1.3.4 *Phialophora graminicola*

Phialophora is a form genus composed of dematiaceous hyphomycetes with philaides possessing collarettes, borne singly or in small clusters. (*Phialocephala* differs from *Phialophora* in that it produces phialides in a dense head composed of multiple metulae.) *Phialophora finlandia* is widely accepted as a DSE fungus (section 1.2.3), but other species of *Phialophora* also fit the criteria for inclusion in the DSE. One of these, *Phialophora graminicola* (Deacon) Walker, is briefly discussed here.

Phialophora graminicola (syn. *Phialophora radicicola* var. *graminicola* Deacon) is the anamorph of *Gaeumannomyces cylindrosporus* (Magnaporthaceae) (Walker 1981). Colonies are compact, pale grey to greenish-grey (Deacon 1974). In its anamorphic phase, phialides are borne on a branched condiophore and are variable in size (7-18 µm X 2-3 µm), tapered toward the apex with a collarette, and produce conidia, 4.5 X 2 µm, variable in shape, usually ellipsoidal, and occasionally crescent-shaped (Deacon 1974). In its sexual state, *Gaeumannomyces cylindrosporus* produces globose black perithecia, 280-565 µm diam. (Hornby *et al.* 1977). Asci are clavate, 65-135 X 9-16 µm, tapering but rounded at apex, produced on a stalk up to 24 µm long, and evanescent when mature (Hornby *et al.* 1977). Eight ascospores are produced per ascus; ascospores are hyaline, cylindrical, slightly curved, commonly 3-4 septate but sometimes up to 8-septate, 37-69 X 3.2-5.6 µm, with rounded tapered ends (Hornby *et al.* 1977).

Phialophora graminicola often colonizes the root cells of grasses and cereals in agricultural fields (e.g., Deacon 1974; Hornby *et al.* 1977) where it is avirulent (Landschoot & Jackson 1990) and acts as an antagonist to the causal agent of take-all disease, *Gaeumannomyces graminis* (Deacon & Henry 1981). Intracellular colonization of the roots of a grass, *Vulpia ciliata*, by *Phialophora graminicola* increases the seedling growth of the host plant (Newsham 1999).

1.3.5 Retention of the term "DSE"

The dark septate root endophytes vary widely in colonial characterisitics, type of association formed with plant roots, and morphology of conidiogenous structures (where formed). Further, they are phylogenetically diverse and disjunct, with little connecting them other than the presence of dark, septate hyphae and the ease with which they are isolated from the roots of plants in a wide range of habitats. However, the retention of the term "DSE" is advocated here because of the ubiquitous presence of these fungi in plant roots and the usefulness of the term for referring to unidentified dark septate fungi observed in direct mounts and in unidentified cultures (e.g. Summerbell 1989, Bledsoe *et al.* 1990, Shishido *et al.* 1996, Arosena & Glowa 2000.)

1.4 Distribution of DSE fungi

DSE fungi are circumboreal in distribution (Jumpponen & Trappe 1998a), isolated from or directly observed in the roots of both angiosperms and gymnosperms, from North America, Europe and Asia, and from a wide variety of habitat types including boreal forests (e.g. Ahlich *et al.* 1998, Visser 1995), bogs (Thormann *et al.* 1999) and other wetlands (Thormann *et al.* 1999, Addy *et al.* 2000), agricultural fields (Narisawa *et al.* 2002), rangelands (Barrow & Aaltonen 2001), and alpine and arctic forests, heaths and meadows (Haselwandter 1987, Bledsoe *et al.* 1990, Stoyke & Currah 1991). Within this larger distribution some patterns have been noted. For example, DSE fungi appear to become more frequent with increasing altitude (Stoyke and Currah 1991), and Haselwandter and Read (1982) have suggested that some DSE fungi play an important ecological role in high altitude environments by increasing phosphorus uptake. A similar abundance of DSE fungi then should be expected in arctic habitats, since arctic and alpine tundra bear many similarities such as low temperature, dominance of ericaceous plants, and low availability of soil nutrients (Gardes & Dahlberg 1996). Kohn and Stasovski (1990) found relatively few DSE in their examination of the roots of plants from Ellesmere Island, Nunavut, but Bledsoe *et al.* (1990) found DSE fungi in the roots of plants on Devon Island, Nunavut. These observations prompted me to examine the distribution of DSE along a latitudinal transect in Canada running from the high arctic to the 49th parallel.

Although it would be useful to know approximately how the frequency of DSE associations changed along this transect, I was especially interested in whether the distribution of individual DSE fungal species exhibit unique patterns. Thus, I isolated and identified, or characterized as much as possible, DSE fungi from the roots of a range of *Salix* species along a latitudinal transect in Canada from 49°N to 78°N. *Salix* was chosen as the target host because it was my objective to keep the host plant as consistent as possible, and although species sampled will change with latitude, *Salix* is the only genus of woody plant found across the entire transect. It was expected that DSE would be isolated consistently across the latitudinal transect but that fungal species composition within these suites of isolates would vary with latitude. This line of investigation is reported in Chapter 2.

1.5 Patterns of genetic variation in *Phialocephala fortinii*, a representative DSE species, across a latitudinal gradient

A second objective in this project was to examine in more detail the genetic variation of *Phialocephala fortinii*, a taxon expected to be the most common and easily identified representative of the DSE fungi along the transect. Pursuit of this objective was motivated by the need to determine if the array of genetic variation seen in previous studies could be attributed to a sampling artifact. In most cases (Stoyke *et al.* 1992, Jumpponen 1999, Grünig *et al.* 2001, Grünig *et al.* 2002), *Phialocephala fortinii* has been observed to be genetically highly variable, with 21 unique inter-simple-sequence-repeat-anchored polymerase chain reaction (ISSR-PCR) genotypes found within a 3 m X 3 m forest plot (Grünig *et al.* 2002) and multiple individuals isolated from the same host plant (Jumpponen 1999, Grünig *et al.* 2002).

Observable genetic differentiation among strains, based on geographical distance as well as on host and habitat variables, is expected. However, in multiple studies using a variety of molecular techniques to assess genetic variation in *Phialocephala fortinii*, host species, habitat and geographical distance did not correlate with genetic variation in this species. Stoyke *et al*. (1992) examined restriction fragments of amplified ribosomal DNA (PCR/RFLP) of eight *Phialocephala fortinii* isolates from a single alpine site and failed to find a relationship between genetic variation in strains of *P. fortinii* and the plants from which they were isolated. In another PCR/RFLP study, Addy *et al.* (2000) examined 33 isolates of *P. fortinii* collected from two 300 m transects and failed to draw a correlation among colony morphology and habitat type, and RFLP-based genetic fingerprints. Using randomly amplified polymorphic DNA (RAPD) analysis, Jumpponen (1999) examined 34 isolates from two areas of approximately 12 m^2 and 112 m^2, and failed to find a pattern in genetic variation which could be attributable to physical distance between isolates. Grünig *et al.* (2001) used ISSR-PCR to examine genetic variation in 14 strains of *P. fortinii* from Switzerland and Germany and failed to find a correlation between ISSR-PCR-based genetic fingerprints and either host plant species or geographical origin of strains.

In past studies examining patterns of genetic variation in *P. fortinii* sampling has either been restricted to a small area with multiple individuals (Stoyke *et al.* 1992, Jumpponen 1999, Addy *et al.* 2000, Grünig *et al.* 2002) or to a large sample area but only with one or two individuals per location (Grünig *et al.* 2001). It is possible that the failure to find a correlate to genetic variation is due to restricted sample sizes, which may not be representative of the populations sampled, or to the small geographical area sampled, which may not fully represent the population being sampled. Further, none of these studies sampled multiple individuals from more than one or two geographic locations.

The objective of Chapter 3 was to characterize genetic variation of *Phialocephala fortinii* and to determine if variation over a much wider area than examined before, and across a broad latitudinal gradient, would yield patterns not discernable in the smaller scale studies descibed above.

1.6 Literature cited

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Table 1.1. Isolation history, geographic distribution and types of associations formed by selected species of dark septate root endophytes (sorted by date of isolation).

Taxon	Source	Location	Morphology of association with host roots[1]	Reference / accession number[2]
Phialocephala dimorphospora Kendrick	Rotten deciduous wood	Ontario, Canada	N/A	Kendrick 1961
	Rotten deciduous wood	Québec, Canada	N/A	Kendrick 1961
	Slime in pulp mill	New Brunswick, Canada	N/A	No reference / UPSC-2186
	Forest soil	Cheshire, England	N/A	Gams 1963
	Sphagnum peat	Seefeld-Tyrol, Austria	N/A	Gams 1963
	Not noted	Germany	N/A	CBS 976.72
	Picea mariana short root	Quebec, Canada	Not noted	Richard & Fortin 1973
	Pediatric isolate of chemotherapy patient	Manitoba, Canada	N/A	No reference / UAMH-8326
Phialocephala fortinii Wang & Wilcox	*Pinus sylvestris* root	Suonenjoki, Finland	Pseudomycorrhizal	Wang & Wilcox 1985 / CBS-443.86
	Calypso bulbosa mycorrhizal root	Alberta, Canada	Orchid root endophyte	Currah *et al.* 1988 / UAMH-5424
	Luetkea pectinata root	Alberta, Canada	Pseudomycorrhizal / parasitic	Stoyke & Currah 1991 / UAMH-6677
	Cassiope mertensiana root	Alberta, Canada	Not noted	Stoyke & Currah 1991 / UAMH-6814
	Lupinus latifolus root	Oregon, USA	Pseudomycorrhizal	O'Dell & Trappe 1992

	Kalmia microphylla root	British Columbia, Canada	Not noted	Currah & Tsuneda 1993 / TMI-32109
	Rhododendron obtusum root	Tottori, Japan	Not noted	Currah & Tsuneda 1993 / TMI-32110
	Abies alba root tip	Jura, Switzerland	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996 / CBS-109310
	Fagus sylvatica root tip	Jura, Switzerland	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996
	Fagus sylvatica root tip	Zürichberg, Switzerland	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996
	Picea abies root tip	Harz, Germany	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996 / CBS-109311
	Picea abies root tip	Bödmerenwald, Switzerland	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996 / CBS-109307
	Picea abies root tip	Valle Maggia, Switzerland	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996 / CBS-109312
	Picea abies root tip	Mesocco, Switzerland	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996
	Picea abies root tip	Säntimassiv, Switzerland	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996
	Picea abies root tip	Selva, Switzerland	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996
	Pinus sylvestris root tip	Kevo, Finland	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996
	Pinus sylvestris root tip	Kneseback, Germany	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996
	Pinus sylvestris root tip	Zürichberg, Switzerland	Not noted / non-ectomycorrhizal	Ahlich & Sieber 1996
	Salix glauca root	Alberta, Canada	Pseudomycorrhizal / ectomycorrhizal	Fernando & Currah 1996 / UAMH-8148
	roots of *Pinus strobus* seedlings	Ontario, Canada	Not noted	UAMH-8321
	Erica herbacea root	Smrjene, Slovenia	Not noted	Vodnik *et al.* 1997 / UAMH-8433
	Andromeda polifolia root	Alberta, Canada	Not noted	Hambleton & Currah 1997

	Cassiope mertensiana root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Cassiope tetragona root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Chamaedaphne calyculata root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Empetrum nigrum root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Gaultheria humifusa root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Kalmia polifolia root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Loiseleuria procumbens root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Menziesia ferruginea root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Phyllodoce empetriformis root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Phyllodoce grandiflora root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Rhododendron albiflorum root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Vaccinium membranaceum root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Vaccinium myrtilloides root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Vaccinium scoparium root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Vaccinium uliginosum root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Vaccinium vitis-idaea root	Alberta, Canada	Not noted	Hambleton & Currah 1997
	Phyllodoce empetriformis root	Washington, USA	Not noted	Jumpponen 1999
	Salix commutata root	Washington, USA	Not noted	Jumpponen 1999

	Juncus sp. root	Washington, USA	Not noted	Jumpponen 1999
	Luzula piperi root	Washington, USA	Not noted	Jumpponen 1999
	Cassiope mertensiana root	Washington, USA	Not noted	Jumpponen 1999
	Vaccinium deliciosum root	Washington, USA	Not noted	Jumpponen 1999
	Arctostaphylos uva-ursi root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Fragaria virginiana root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Galium boreale root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Geocaulon lividum root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Maianthemum canadense root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Ledum groenlandicum root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Oxycoccus microcarpus root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Linnaea borealis root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Carex gynocrates root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Betula pumila root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Menyanthes trifolia root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Salix pedicellaris root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Smilacina trifolia root	Alberta, Canada	Not noted	Addy *et al.* 2000
	Betula platyphylla var. *japonica* root	Mt. Norikura, Japan	Mycorrhizal endophyte	Hashimoto & Hyakumachi 2001 / UAMH-9523

	Pinus sylvestris root	Kevo, Finland	Not noted	Grünig *et al.* 2001 / CBS-109300
	Forest soil	Oberschlatt, Switzerland	N/A	Grünig *et al.* 2001 / CBS-109302
	Forest soil	Zernez, Switzerland	N/A	Grünig *et al.* 2001 / CBS-109303
	Forest soil	Martigny, Switzerland	N/A	Grünig *et al.* 2001 / CBS-109304
	Forest soil	Isérable, Switzerland	N/A	Grünig *et al.* 2001 / CBS-109305
	Forest soil	Offenpass, Switzerland	N/A	Grünig *et al.* 2001 / CBS-109306
	Calluna vulgaris root	Amden, Switzerland	Not noted	Grünig *et al.* 2001 / CBS-109313
	Solanum melongena root	Shizuoka, Japan	Pseudomycorrhizal	Narisawa *et al.* 2002
	Cucumis melo root	Shizuoka, Japan	Not noted	Narisawa *et al.* 2002
	Lycopersicon esculentum root	Shizuoka, Japan	Not noted	Narisawa *et al.* 2002
	Fragaria grandiflora root	Shizuoka, Japan	Not noted	Narisawa *et al.* 2002
Phialophora finlandia Wang & Wilcox	Root of *Pinus sylvestris* seedling	Suonenjoki, Finland	Ectendomycorrhizal	Wang & Wilcox 1985 / CBS-444.86
	Root tip of *Pinus strobus* seedling	Ontario, Canada	Ectendomycorrhizal	Schelkle *et al.* 1996 / UAMH-8322
	Arctostaphylos uva-ursi root	Alberta, Canada	Not noted	Hambleton, pers. comm.
Chloridium paucisporum Wang & Wilcox	Short root of *Pinus resinosa* seedling	New York, USA	Ectendomycorrhizal	Wang & Wilcox 1985 / CBS-445.86
Leptodontidium orchidicola Sigler & Currah	*Coeloglossum viride* root	Alberta, Canada	Orchid root endophyte	Currah *et al.* 1987 / UAMH-5420
	Corallorhiza maculata root	Alberta, Canada	Orchid root endophyte	Currah *et al.* 1987 / UAMH-5421

	Calypso bulbosa root	Alberta, Canada	Orchid root endophyte	Currah *et al.* 1988 / UAMH-5441
	Listera borealis root	Alberta, Canada	Orchid root endophyte	Currah *et al.* 1990 / UAMH-6086
	Platanthera orbiculata root	Alberta, Canada	Mycorrhizal symbiont	Currah *et al.* 1990 / UAMH-6087
	Corallorhiza trifida root	Alberta, Canada	Not noted	Fernando & Currah 1995 / UAMH-6085
	Spiranthes lacera roots	Manitoba, Canada	Not noted	Fernando & Currah 1995 / UAMH-8150
	Piperia unalascensis	Alberta, Canada	Not noted	Fernando & Currah 1995 / UAMH-5627
	Platanthera huronensis root	Alberta, Canada	Not noted	Fernando & Currah 1995 / UAMH-5422
	Artemisia norvegica root	Alberta, Canada	Pseudomycorrhizal	Fernando & Currah 1996 / UAMH-8151
	Pedicularis bracteosa root	Alberta, Canada	Mycorrhizal endophyte	Fernando & Currah 1996 / UAMH-8152
	Carex sp. root	Alberta, Canada	Mycorrhizal endophyte	Fernando & Currah 1996 / UAMH-8149
	Trollius albiflorus root	Alberta, Canada	Not noted	Fernando & Currah 1996
	Castilleja sp. root	Western Canada	Not noted	Fernando & Currah 1996
	Rubus sp. root	Western Canada	Not noted	Fernando & Currah 1996
	Erigeron sp. root	Western Canada	Not noted	Fernando & Currah 1996
	Achillea sp. root	Western Canada	Not noted	Fernando & Currah 1996

	Heracleum lanatum root	Western Canada	Not noted	Fernando & Currah 1996
	Abies balsamea root	Western Canada	Not noted	Fernando & Currah 1996
	Platanthera dilatata root	Alberta, Canada	Root endophyte	Harney *et al.* 1997, Addy *et al.* 2000 / UAMH-5628
	Betula platyphylla var. *japonica* root	Mt. Norikura, Japan	Not noted	Addy *et al.* 2000 / UAMH-9524
Heteroconium chaetospira (Grove) Ellis	Wheat field soil	Schelswig-Holstein, Germany	N/A	No reference / CBS-514.63
	Agricultural soil	Wageningen, The Netherlands	N/A	No reference / CBS-755.68
	Picea abies root	Denmark	Not noted	No reference / CBS-491.70
	Ammophila arenaria root	United Kingdom	Not noted	No reference / IMI-60911
	Human skin	West Australia	N/A	No reference / IMI-236020
	Soil	India	N/A	No reference / IMI-244929
	Pinus sylvestris needle	Baarn, The Netherlands	N/A	No reference / CBS-466.82
	Pseudotsuga menziesii root	Veluwe, The Netherlands	Not noted	No reference / CBS-215.86
	Solanum melongena root	Shizuoka, Japan	Non-pathogenic	Narisawa *et al.* 2002
	Cucumis melo root	Shizuoka, Japan	Not noted	Narisawa *et al.* 2002
	Lycopersicon esculentum root	Shizuoka, Japan	Not noted	Narisawa *et al.* 2002
	Fragaria grandiflora root	Shizuoka, Japan	Not noted	Narisawa *et al.* 2002

Hymenoscyphus ericae (Read) Korf & Kernan	Roots of Ericaceae	Sheffield, England	Root endophyte	Read 1974 / UAMH-6513
	Calluna vulgarisi root	Bolsterstone, UK	Root endophyte	Pearson & Read 1973 / UAMH-6735
	Vaccinium angustifolium root	Quebec, Canada	Root endophyte	Couture *et al.* 1983 / UAMH-6598
	Vaccinium corymbosum root	Quebec, Canada	Root endophyte	Couture *et al.* 1983 / UAMH-6663
	Vaccinium angustifolium root	Maine, USA	Root endophyte	Dalpé *et al.* 1989 / CBS-652.89
	Leucopogon parviflorus root	New South Wales, Australia	Ericoid mycorrhizal	Steinke *et al.* 1996
	Andromeda polifolia root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Cassiope mertensiana root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Cassiope tetragona root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Chamaedaphne calyculata root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8873
	Empetrum nigrum root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8865
	Gaultheria humifusa root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Kalmia polifolia root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Rhododendron groenlandicum root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8680
	Loiseleuria procumbens root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Menziesia ferruginea root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Oxycoccum quadripetalus root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8872

	Phyllodoce empetriformis root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8867
	Phyllodoce grandiflora root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Rhododendron albiflorum root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Vaccinium membranaceum root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Vaccinium myrtilloides root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8869
	Vaccinium scoparium root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Vaccinium uliginosum root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Vaccinium vitis-idaea root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8870
	Cephaloziella exiliflora rhizoids	Antarctica	N/A	Chambers *et al.* 1999
	Calluna vulgaris root	England	Ericoid mycorrhizal	Sharples *et al.* 2000
Oidiodendron maius Barron	Peat soil (bog)	Ontario, Canada	N/A	Barron 1962 / UAMH-1540
	Spruce wood	Ontario, Canada	N/A	Barron 1962
	Forest soil	Ontario, Canada	N/A	Barron 1962
	Root of Ericaceae	Sweden	Root endophyte	No reference / CBS-334.52
	Forest soil	Sweden	N/A	No reference / CBS-926.73
	Forest soil, under *Abies spectabilis* and *Betula utilis*	Western Nepal	N/A	No reference / CBS-457.74

	Vaccinium corymbosum	Quebec, Canada	Ericoid mycorrhizal	Couture *et al.* 1983 / UAMH-8529
	Coniferous forest soil	Gusum, Sweden	N/A	Nordgren *et al.* 1985
	Picea sitchensis ectomycorrhizal root tip	Ireland	Not noted	Schild *et al.* 1988
	Rhododendron sp. root	Ireland	Ericoid mycorrhizal	Douglas *et al.* 1989
	Loiseleuria procumbens	Alberta, Canada	Ericoid mycorrhizal	Stoyke & Currah 1991 / UAMH-6514
	Gaultheria shallon	British Columbia, Canada	Ericoid mycorrhizal	Xiao & Berch 1992 / UAMH-7022
	Soil under *Fagus sylvatica*	Switzerland	N/A	No reference / IMI-184624
	Leaf litter of *Buchenavia capitata*	Cuba	N/A	No reference / CBS-247.95
	Calluna vulgaris root	Not noted	Ericoid mycorrhizal	Perotto *et al.* 1996
	Andromeda polifolia root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Cassiope mertensiana root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Cassiope tetragona root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Chamaedaphne calyculata root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8919
	Empetrum nigrum root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Gaultheria humifusa root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Rhododendron groenlandicum root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8932

	Loiseleuria procumbens root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Oxycoccus quadripetalus root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8920
	Phyllodoce emeptriformis root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8933
	Phyllodoce glanduliflora root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8923
	Rhododendron albiflorum root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Vaccinium myrtilloides root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8921
	Vaccinium scoparium root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8924
	Vaccinium uliginosum root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997
	Vaccinium vitis-idaea root	Alberta, Canada	Ericoid mycorrhizal	Hambleton & Currah 1997 / UAMH-8922
	Woolsia pungens root	New Zealand	Ericoid mycorrhizal	Liu *et al.* 1998
	Vaccinium myrtillus root	Poland	Ericoid mycorrhizal	Lacourt *et al.* 2000
	Rotting *Picea glauca* log	Alberta, Canada	N/A	Lumley *et al.* 2001
	Sphagnum fuscum	Alberta, Canada	N/A	Thormann *et al.* 2001
	*Empetrum nigrum*root	Kevo, Finland	Ericoid mycorrhizal	Currah *et al.* 1999
	Vaccinium myrotillus root	Kevo, Finland	Ericoid mycorrhizal	Currah *et al.* 1999

	Vaccinium vitis-idaea root	Kevo, Finland	Ericoid mycorrhizal	Currah *et al.* 1999
	Vaccinium angustifolium root	Pennsylvania, USA	Ericoid mycorrhizal	No reference / UAMH-9263
	Vaccinium vitis-idaea root	Dwingelderveld, Netherlands	Root endophytic	No reference / CBS-110450
Phialophora graminicola (Deacon) Walker	Root of *Triticum* sp.	UK	Not noted	IMI 224174
	Grass root	UK	Not noted	Hornby *et al.* 1977 / CBS-609.75
	Poa pratensis root	New York, USA	Not noted	Smiley & Fowler 1984
	Poa annua root	Rhode Island, USA	Not noted	Jackson & Landschoot 1986
	Grass root	Poland	Not noted	Martyniuk 1986
	Triticum aestivum root	USA	Not noted	Wiese 1987
	Poa pratensis root	Rhode Island, USA	Not noted / antagonistic to root pathogens	Landschoot & Jackson 1990
	Lolium perenne root	Rhode Island, USA	Not noted / antagonistic to root pathogens	Landschoot & Jackson 1990
	Vulpia ciliata ssp. *ambigua* root	UK	Not noted / beneficial to plant	Newsham 1999

[1] In cases where taxa were isolated from material other than roots, N/A indicates not applicable.

[2] CBS = Centraalbureau voor Schimmelcultures, The Netherlands; IMI = International Mycological Institute, United Kingdom; TMI = Tottori Mycological Institute, Japan; UAMH = University of Alberta Microfungus Collection and Herbarium, Canada; UPSC = Uppsala University Culture Collection, Sweden.

Chapter 2: Distribution and identification of dark septate root endophytic (DSE) fungi along a latitudinal transect in Canada

2.1 Introduction

2.1.1 Definition of DSE

Dark septate root endophytes (DSE) are an artificial assemblage of fungi that have darkly pigmented septate hyphae and that are frequent or distinctive intracellular associates of roots of apparently healthy plants. DSE fungi have been reported from over 600 species of plants from 114 families (Jumpponen & Trappe 1998) and can form a variety of associations with host plants, including ectendomycorrhizas, pseudomycorrhizas and ericoid mycorrhizas (e.g. Wilcox *et al.* 1974, Wang & Wilcox 1985, Read 1996). Some strains may act as mutualists by increasing the dry weight of host plants (Wang & Wilcox 1985, Fernando & Currah 1996), while others act as parasites by killing hosts or decreasing their dry weight (Melin 1922, Wilcox & Wang 1987, Stoyke *et al.* 1993). Still others do not appear to produce any effect on host plants when inhabiting their roots (Stoyke & Currah 1991, O'Dell *et al.* 1993).

In addition to being isolated from roots, many DSE fungal taxa are also known from forest soils (Gams 1963, Grüning *et al.* 2001), agricultural soils (Deacon 1974), peat (Barron 1962, Gams 1963, Thormann *et al.* 2001) and rotting wood (Kendrick 1961, Lumley *et al.* 2001). They display a range of enzymatic abilities (e.g. Perotto *et al.* 1997, Caldwell *et al.* 2000, Bartholdy *et al.* 2001, Rice & Currah 2001); these fungi likely possess the ability to act as both saprophytes and mutualists. Identified species of DSE fungi include *Phialocephala fortinii*, *Phialocephala dimorphospora*, *Leptodontidium orchidicola*, *Chloridium paucisporum*, *Phialophora finlandia*, *Phialophora graminicola*, and *Exophiala jeanselmei*, among others (see Chapter 1).

2.1.2 Distribution of DSE fungi

DSE fungi are frequently isolated from plants in most cold- and nutrient-stressed habitats (Richard & Fortin 1974, Haselwandter & Read 1982) and in soils with high organic content (Fernando & Currah 1996). They are circumboreal in distribution (Jumpponen & Trappe 1998), and have been reported from North America, Europe and Asia in various habitats including boreal forests, montane forests, bogs and other wetlands, agricultural soil, prairies, and alpine and arctic tundra. It has been hypothesized that DSE play an important role in alpine systems by increasing nutrient uptake (Haselwandter & Read 1982), but their presence in arctic systems has been assessed rarely (Kohn *et al.* 1990, Bledsoe *et al.* 1990) and the nature of their association in the arctic has not been investigated.

Although DSE fungi appear to be ubiquitous in circumboreal regions, distribution patterns of individual DSE species are difficult to ascertain since many DSE fungi remain unidentified (e.g. Bledsoe *et al.* 1990, Shishido *et al.* 1996, Arosena & Glowa 2000). In studies where DSE fungi have been identified to species, limited geographic areas have been considered (e.g. Jumpponen 1999, Addy *et al.* 2000). An examination of the diversity of DSE fungi across a wide geographic area may reveal broad distribution patterns of DSE species that have not been observed in smaller scale studies.

2.1.3 Objective

The objective of this study was to isolate and identify or describe the species of DSE fungi in a single host taxon (i.e., *Salix*) along a transect running from short grass prairie at 49°N through the boreal forest (56°N and 62°N) to the high arctic at 78°N. It was expected that DSE fungi would be found inhabiting the roots of plants sampled at each point along the latitudinal gradient, but that species assemblages would differ.

2.2 Materials and methods

2.2.1 Study sites

Three sites, at least 1.5 km apart, were chosen at each of four latitudes (Figure 2.1). Latitude One was approximately 200 km north of One Four, Alberta, at 49°N -

110°W. Three sites (RW, RE and C) were sampled May 20-21, 2000. Sites RW and C at Latitude One were grazed areas of short grass prairie; site C was in a slough dominated by *Salix*. Sites FM, B and SP at Latitude Two, approximately 20 km east of Fort McMurray, Alberta, at 56°N - 111°W, and within the boreal forest region, were sampled July 12-13, 2000. Sites N, W and T at Latitude Three, approximately 30 km west of Yellowknife, Northwest Territories, at 62°N - 114°W, were sampled between July 20 and 23, 2000. Latitude Four at Alexandra Fjord on Ellesmere Island in Nunavut, Canada, 78°N - 75°W, is a "high arctic oasis." This area has a slightly higher average temperature and soil moisture in relation to the surrounding region because of high cliffs on either side of the sites that trap heat, and sites are on a relatively flat plain that continuously receives glacial runoff water during the growing season. Sites M, K and D were sampled at Latitude Four between July 29 and 31, 2000.

At each site, eight *Salix* plants were selected by stratified random design: A 40 m X 40 m plot was created, divided into four 20 m X 20 m quadrats, and two *Salix* plants were selected at random within each quadrat, regardless of age, species, or condition of the plant. *Salix* was selected because it is the only genus of woody plant found at each site along the latitudinal transect. Climate data at each latitude are summarized in Appendix 1, and plant species found at each site in Appendix 2.

2.2.2 Isolation of fungi from *Salix* roots

Three contiguous root segments (two 5 mm segments and one approx. 2 cm segment) were excised from each *Salix* plant approximately 30 cm from the main stem. The 2 cm segment was immersed in 5% KOH and autoclaved at 121°C for 15 min to clear root cells (Philips & Hayman 1970), then mounted in glycerol to be examined for dark septate hyphae within and between root cells, microsclerotia, and mantle tissue.

The remaining two 5 mm root segments were surface sterilized for 2 min in commercial (5.25%) hypochlorite bleach : dd-H_2O (1:2), and cut into approximately 1 mm bits. To maximize recovery of target fungi, root bits were plated onto both corn meal agar with oxytetracycline [CMA+t; 15 g corn meal agar (Fisher Scientific B11132), 10 mg oxytetracycline dihydride (Sigma O-5750), 1 L dd-H_2O] and CMA+t with rose bengal disodium salt (33 mg / L; Sigma R-3877), and incubated at 23°C for two months. Dark colonies of sterile septate hyphae were isolated and stored at 4°C for 4 to 15 months. Hyaline colonies were isolated only when they were distinctive in their pattern of occurrence or morphology. Colonies were plated onto CMA [15 g corn meal agar (Fisher Scientific B11132), 1 L dd-H_2O], and / or malt extract agar [MEA; 15 g malt extract (Difco 218630), 20 g Bacto® agar (Difco 214010), 1 L dd-H_2O], modified Melin Norkan's agar (MMN; Marx 1969), green bean extract agar [GBA; 98g Gerber's strained green bean baby food, 20 g Bacto® agar, 1 L dd-H_2O], and / or modified Leonian's agar [MLA; 6.25 g maltose (Sigma 47288), 6.25 g malt extract (Difco 218630), 1.25 g KH_2PO_4, 1.0 g yeast extract (Difco 212750), 0.625 g $MgSO_4 \cdot 7H_2O$, 0.625 g Bacto® peptone (Difco 211840), 20 g Bacto® agar (Difco 214010), 1 L dd-H_2O].

2.2.3 Identification of fungal isolates and phylogenetic analyses

Sporulating strains were identified on the basis of conidiophore and conidium morphology. A subset of both sterile strains and sporulating strains was grown on tap water agar [TWA; 15 g Bacto® agar (Difco 214010), 1 L dd-H_2O] and single hyphal tips were used to produce pure colonies on CMA. Pure strains were grown in still liquid cultures of potato dextrose broth [PDB; 15 g potato dextrose broth (Difco 254920), 1 L dd-H_2O] at 14°C for 2 weeks. Mycelia were rinsed in sterile dd-H_2O to remove residual PDB, and then lyophilized. Total genomic DNA was extracted as follows: 0.02 g lyophilized fungal tissue was ground in sand with approximately 0.002 g polyvinylpolypyrrolidone (PVPP; Sigma P-6755), then added to 750 µl 2X CTAB buffer (Doyle & Doyle 1987) with 0.2 µl ß-mercaptoethanol (Sigma M-7154) and incubated at 65°C for 1 h. Proteins were removed by adding and mixing 750 µl 24 : 1 chloroform : isoamyl alcohol to the CTAB solution, and then centrifuging at 13 200 rcf for 20 min. The supernatant was removed and DNA was purified using a QIAquick PCR Purification Kit (Qiagen 28104): 3.5 ml Buffer PB was added to DNA, mixed, and run through a purification column; bound DNA was washed with 750 µl Buffer PE and eluted with 30 µl 65 °C UV-sterilized dd-H_2O. DNA was treated with 0.5 ng RNase A and incubated at 37 °C for 30 min and stored at –20°C.

The second internal transcribed spacer (ITS2) region of ribosomal DNA was amplified using prITS3 and prITS4 (White *et al.* 1990) with a PE GeneAmp 9700 thermal cycler using standard reaction conditions. The thermal profile for the PCR reactions was: 35 cycles of: 1 min at 94°C, 1 min of 55°C and 2 min at 72°C, with an initial denaturation of 94°C for 2 min and a final extension of 72°C for 7 min. PCR products were visualized using 1.5% agarose gel electrophoresis. The ITS2 region was sequenced using the DYEnamic™ ET Terminator cycle sequencing kit (Amersham Pharmacia) with each of the primers used for amplification, with the following cycling parameters: 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; and visualized with an ABI 377 sequencer. Sequencher 3.0 (Gene Codes Corp., Ann Arbor, Michigan, USA) was used to compile contiguous sequences from resulting electrophoregrams. PCR primer sequences were excluded from contigs, and completed contigs were exported from Sequencher as text files.

The first phylogenetic analysis comprised known Leotialean fungi and sterile strains thought to belong to the Leotiales: 23 sporulating strains of *Phialocephala fortinii*, 13 sterile strains suspected to be *P. fortinii*, four sterile strains suspected to be *Leptodontidium orchidicola*, and one sterile strain similar to *Phialophora*. ITS2 sequences from *Hymenoscyphus ericae*, *Leptodontidium orchidicola*, *Phialocephala compacta*, *Phialocephala dimorphospora*, *Phialocephala fortinii*, *Phialocephala scopiformis*, *Phialophora finlandia*, *Phialophora gregata* and an unnamed dark septate endophyte (DSE DS16b, Schadt *et al.* 2001) from NCBI GenBank were also included in the analysis (Table 2.1).

Sequences were aligned with CLUSTAL W (Thompson *et al.* 1994). Alignments were exported in PHYLIP format (Felsenstein 1995), then imported into Se-Al 1.0 (Rambaut 1998) for minor adjustment following Graham *et al.* (2000). Final alignment was exported as a NEXUS format and imported into PAUP * v. 4.10b (Swofford 1999).

An heurstic search was performed in PAUP* v. 4.10b with tree-bisection-reconnection (TBR) branch swapping and default settings. A maximum of 10 000 trees was set to limit analysis time. Bootstrap analysis (Felsenstein 1985) was performed on a reduced data set (all *Phialocephala fortinii* sequences were excluded except strains D3 and N6 from this study, and AF214583 from GenBank) with 1000 replicates. A "fast" bootstrap analysis (1000 replicates) was also performed with all taxa included. Trees were rooted with *Hymenoscyphus ericae* (Grünig *et al.* 2002a).

The second phylogenetic analysis included one strain (C7) suspected to be a species of *Exophiala* (Herpotrichiellaceae). An alignment of ITS1, 5.8S and ITS2 segments of rDNA for 26 Herpotrichiellacean species (14 species of *Capronia*, a teleomorphic genus, and 12 anamorphic species) was supplied by W. Untereiner (Table 2.1). This alignment was imported into Se-Al 1.0 (Rambaut 1998), then the ITS2 sequence for strain C7 was inserted into the alignment and adjusted following Graham *et al.* (2000). ITS1 and 5.8S sequences were retained for Herpotrichiellacean taxa, and were left as missing for strain C7 in subsequent analysis. Final alignment was exported as a NEXUS format and imported into PAUP * v. 4.10b (Swofford 2002).

An heurstic search was performed in PAUP* v. 4.10b with TBR branch swapping and default settings. Bootstrap analysis (Felsenstein 1985) was performed with 100 replicates. Trees were rooted with *Capronia villosa* and *Capronia dactylotricha* (Untereiner & Naveau 1999).

2.3 Results

2.3.1 DSE structures in squash mounted roots of *Salix* species

Evidence of DSE fungi was observed in 81 of 96 squash-mounted root samples (Figures 2.2, 2.3, 2.4 and 2.5); nine root samples were destroyed during the clearing process (Table 2.2), and six root segments examined (all from 49°N) were devoid of any dark fungal structures. Dematiaceous septate hyphae were present in 16 of 22 root segments examined from 49°N, sclerotia were only observed in two segments, and mantle tissue was not observed. Dark septate hyphae were observed in all roots segments examined from 56°N, 62°N and 78°N, and microsclerotia were observed in 11 of 23 roots examined from 56°N, 12 of 20 root segments examined from 62°N, and 9 of 22 root segments from 78°N. Black mantle tissue was observed in 13 of 23 roots examined from sites at 56°N, 6 of 20 roots from sites at 62°N, and 13 of 22 roots from sites at 78°N.

2.3.2 Dark, septate fungi isolated from *Salix* roots

Root endophytic fungi with dark, septate hyphae were recovered from *Salix* at all latitudes (Table 2.2). Fungi were identified on the basis of conidium morphology, or, where conidia were absent, divided among eight groups based on cultural characteristics. Four of these groups were eventually allied with *Phialocephala fortinii*, one with *Leptodontidium orchidicola*, and one with *Phialophora* by phylogenetic analysis of ITS2 sequence data, and two were not included in the phylogenetic study.

Phialocephala fortinii Wang & Wilcox

Colonies were identified by morphology of conidiogenous cells when produced, or by analysis of ITS2 sequence.

Microscopic features of *Phialocephala fortinii* (Figure 2.6):

Aerial hyphae dematiaceous, septate, 2-3 μm diam., asperate, covered with small, irregular, rounded to flattened blisters. Submerged hyphae sometimes monilioid, 6-8 μm in diam. Conidiophores 5 to 100 μm in length. Conidiogenous heads with 0 to 5 series of metulae. Hyphal loops occasionally present, up to 40 μm diam. Conidiogenous cells 15 X 2-4 μm. Conidiogenous apparati often sterile, staining deeply with acid fuschin. Conidia either abundant, forming loose aggregations at the tip of the conidiophore and staining deeply with acid fuschin, or produced singly, remaining attached to the phialide and non-staining. Conidia hyaline, globose and minute, 1.5 μm diam.

Five different cultural types observed on MMN (Table 2.3), differentiated by colour, texture of aerial mycelium, differences in morphology of margins, and presence or absence of sclerotia.

PF cultural type 1: Mycelium dark brown or dark brown becoming light brown or grey/brown in center, aerial hyphae abundant. Mycelium felt-like. Margin submerged, diffuse and feathery in face view.

PF cultural type 2: Mycelium dark brown with few aerial hyphae in center, becoming completely submerged toward margin, and surrounded by diffuse feathery growth. Reverse black in center, lightening to dark brown at the margin.

PF cultural type 3: Mycelium uniformly dark brown with abundant aerial hyphae, giving a felty appearance. Margins dark brown, surrounded by compact feathery growth. Reverse black to dark brown.

PF cultural type 4: Mycelium medium tan, darkening to dark brown in the center, with diffuse feathery tan growth extending from margin. Reverse is light grey / brown in center, darkening near margin.

PF cultural type 5: Mycelium mouse grey in center, becoming submerged and dark brown at margins which are surrounded by a halo of feathery growth, and large black submerged sclerotia. Reverse uniformly dark brown to black.

Isolated from: Lat. One - 49°N: RW8, C3; Lat. Two - 56°N: FM3, FM6, FM7, FM8, B1, B2, B3, B4, B5, B7, B8, SP3, SP6, SP7; Lat. Three - 62°N: N1, N2, N3, N4, N6, N7, N8; W1, W2, W3, W4, W5, W6, W7, W8; T1, T2, T3, T4, T5, T6, T7, T8; Lat. Four - 78°N:

M1, M2, M3, M4, M5, M6, M8, D1, D2, D3, D4, D5, D6, D7, D8, K2, K3, K5, K6, K7, K8

Leptodontidium orchidicola Sigler & Carmichael

On MEA, mycelium glabrous, dark brown and reverse black. Outer surface of aerial hyphae asperate or smooth. Submerged hyphae sometimes monilioid, 5 µm diam. at widest point. Occasionally hyphal loops produced, up to 30 µm diam. Conidia teardrop shaped, 2.5 X 3.5-4 µm, produced singly and borne directly on hyphae.

Isolated from: Lat. Two - 56°N: FM1, FM2, B4, B6, SP4, SP5; Lat. Four – 78°N: K5 (Only strain from site B4 sporulated in culture).

Exophiala sp. (Figures 2.7 and 2.8)

On MEA mycelium almost completely submerged, pale brown, few grey-brown to brown aerial hyphae in center of colony. Hyphae dematiaceous and septate. Aerial hyphae 2-3 µm diam., smooth-walled. Submerged hyphae smooth-walled, some monilioid cells present. Conidiogenous cells developing directly on vegetative hyphae, varying widely in shape and size from short peg-like cells, 2 µm X 5 µm, to large clavate cells up to 5 µm X 15 µm. Brown, single-celled, oblong to ossiform conidia, 3 X 7 µm, forming in clusters, remaining attached to the conidiogenous locus.

Isolated from: Lat. One – 49°N: RE1, RE2, RE3, RE5, RE8, RW2, C2, C3, C7; Lat. Two - 56°N: SP2

Cadophora sp. Conant (Figure 2.9)

Hyphae smooth-walled, dematiaceous, 3 µm diam. Phialidic conidiogenous cells 7-8 X 3-4 µm, borne directly on vegetative hyphae alone or in clusters of 2-3, dark brown and possess a well-defined brown collarette 1.5 µm deep. (These strains were mixed with *Lecanicillium muscarium* and could not be separated, so cultural characteristics could not be accurately described.)

Isolated from: Lat. One – 49°N: C2

Phialophora sp. Medlar

Note: This strain remained sterile. Colony even mouse grey in colour, with abundant aerial mycelium. Analysis of ITS2 sequence data indicates a close match to *Phialophora gregata* (Figure 2.15).

Isolated from: Lat. Four – 78°N: K1

Trimmatostroma sp. Corda (Figures 2.10 and 2.11)

On MEA, mycelium dark brown, submerged. Hyphae dematiaceous, septate; aerial hyphae smooth-walled to asperate, submerged hyphae smooth-walled. Abundant large dark brown to black intercalary chlamydospores, up to 30 µm diam., ranging from

globose to reniform. Abundant smooth to roughly tuberculate conidia, 10 to 38 µm diam.
Isolated from: Lat. One – 49°N: RW1

Sterile DSE 1 (Figure 2.12)
On MMN, compact dark brown mycelium with black reverse; rubbery, glabrous surface with few aerial hyphae and dense feathery black margins. Aerial hyphae regularly septate, 2-3 µm diam., asperate; submerged hyphae monilioid, forming irregular chains of large doliiform to globose cells, 5 to 15 µm diam. at widest point, 5 to 20 µm in length. Conidiogenous cells not observed.
Isolated from: Lat. One – 49°N: RE4, RW1, RW5

Sterile red-staining DSE (Figure 2.13)
Red diffusible exudate. Hyphae, 2-3 µm diam, forming submerged brown mycelium with mouse grey cottony tufts on the surface. Aerial hyphae with numerous light to dark brown, blister-like protrusions (up to 10 µm length, up to 5 µm height) Submerged hyphae dark brown and occasionally monilioid. Conidiogenous cells not observed.
Isolated from: Lat. One – 49°N: C2, C6, C7; Lat. Two – 56°N: FM1, FM7, SP4

Figure 2.14 summarizes the total number of DSE taxa isolated at each latitude, and the total number of root segments at each latitude from which two or more DSE taxa were isolated.

2.3.3 Hyaline fungi from *Salix* roots

Some root segments did not yield DSE fungi even though DSE fungal structures were visible in squash mounts. Immediate growth of hyaline conidial fungi from root bits overgrew DSE fungi and either made isolation of DSE fungi difficult or overgrew DSE fungi within the Petri plate before isolation was possible. Two frequently isolated species of hyaline fungi were noted:

Unidentified yellow-staining endophyte

Produces yellow exudates in mixed culture. When isolated on MEA, CMA, MMN, ceases to form exudate, displaying a colony with fluffy white aerial hyphae at the center and completely submerged at the margins. Conidia oblong, 2 X 4 µm; conidiogenous cells not observed.

Note: Many isolates of dark septate fungi were mixed with this fungus.

Isolated from: Lat. One – 49°N: RE2, RE5, RE6, RW3, RW4, C1, C2; Lat. Two – 56°N: B1, B7, SP2, SP3, SP4; Lat. Three – 62°N: N4, N6, N8, W2, W5; Lat. Four – 78°N: K1, K5, K6, K8

Lecanicillium muscarium (Petch) Zare

On MEA, MMN and CMA, produced a white colony with few aerial hyphae; hyphae 2 µm diam. Abundant oblong conidia, 1 X 4 µm, produced. Conidiogenous cells not observed.

Note: Always found mixed with a DSE isolate as a sterile white submerged fungus.

Isolated from: Lat. One – 49°N: RE1, RE2, RE4, RE5, RE6, RE7, RE8, RW1, RW3, RW4, RW5, C1, C2, C7, C8; Lat. Two – 56°N: FM6, FM7, B4, B7, SP1, SP3; Lat. Three – 62°N: N4, N8, W1, W2, W5, W6, W7, W8, T2, T3, T7, T8; Lat. Four – 78°N: M3, M8, D4, D5, D7, K2, K8

2.3.4 Phylogenetic analyses of ITS2 sequence data

Ten thousand most parsimonious trees were produced with length of 141 steps in the parsimony analysis comprising Leotialean fungi. In all 10 000 trees, 13 sterile strains isolated from *Salix* roots formed a clade with all known *Phialocephala fortinii* included in the analysis, with 87% bootstrap support, and 80% support with "fast" bootstrap (Figure 2.15); all lineages in this clade are very closely related and were intermixed with those that could be identified on the basis of morphology. Four sterile strains formed a clade with *Leptodontidium orchidicola* with 80% bootstrap support; the clade includes the unnamed dark septate endophyte isolated by Schadt *et al.* 2001 (Figure 2.15). The near-identity of ITS2 for the isolates that could not be confirmed as *P. fortinii* or *L. orchidicola* on the basis of morphology with those that could, and the intermingling of these two types in very well supported clades, all lend strong credence to the conspecificity of these strains to *P. fortinii* or to *L. orchidicola* respectively. Isolate K1, originally thought to be *L. orchidicola* on the basis of colony morphology, is allied with *Phialophora gregata* (58% boostrap support) rather than the *L. orchidicola* clade (Figure 2.15).

Nine most parsimonious trees with length of 1806 steps were produced by the analysis of rDNA sequence data from Herpotrichiellacean taxa. Isolate C7 formed a weakly supported (54% bootstrap support) clade with *Fonsecaea pedrosoi*, *Exophiala salmonis* and *Exophiala pisciphila* (Figure 2.16).

2.4 Discussion

2.4.1 Fungal endophytes from *Salix* roots along a latitudinal transect in Canada

Dark septate fungi were isolated from *Salix* roots at all latitudes sampled. The most frequently isolated DSE fungi were *Phialocephala fortinii*, *Leptodontidium orchidicola* and *Exophiala* sp., while *Trimmatostroma* sp., *Phialophora* sp. and *Cadophora* sp. were isolated from only one plant each.

Phialocephala fortinii was isolated from all latitudes sampled, and was the most frequently isolated representative of the DSE fungi from 56°N, 62°N and 78°N. *Phialocephala fortinii* was first described as an associate of *Pinus sylvestris* and has since been isolated from the roots of 52 species of plants (including *Salix*; Addy *et al.* 2000) comprising 18 families, and is circumboreal in distribution. It is known to be culturally variable (Stoyke 1991, Addy *et al.* 2000); here *P. fortinii* displayed five distinct cultural morphologies on MMN varying in colour, texture, and the presence or absence of sclerotia. There is no correlation between cultural morphology on MMN and the latitude from which the strain was isolated, as three cultural morphotypes include individuals from at least two latitudes (Table 2.3).

Short ectomycorrhizal root tips with black mantles and small patches of dark, discontinuous mantle were observed in root squashes from all latitudes except 49°N. *Phialocephala fortinii* has previously been observed to produce discontinuous mantle on *Salix glauca* seedlings *in vitro* (Fernando & Currah 1996) and is likely the causal agent of the discontinuous mantles observed on *Salix* roots here. *Phialocephala fortinii* was isolated from all latitudes sampled, although with greater frequency at higher latitudes, and only twice from 49°N (Table 2.2). There are no known patterns of host plant specificity in *Phialocephala fortinii* (Stoyke *et al.* 1992, Grünig *et al.* 2002b) and thus we consider the effects of host species and plant community type to play a minor role in influencing the recovery rates of *P. fortinii* along our gradient.

Leptodontidium orchidicola was isolated from *Salix* roots at three of four latitudes, missing only from Latitude 1 (49°N). This is the first record of *L. orchidicola* from *Salix* roots and from an arctic location. *Leptodontidium orchidicola* has been previously recorded from the roots of 17 other plant species including orchids (e.g. *Calypso bulbosa*, Currah *et al.* 1988), and herbaceous (e.g. *Carex* sp., Fernando & Currah 1995) and woody plants (e.g. *Rubus* sp. and *Abies balsamea*, Fernando & Currah 1996), from Alberta and Manitoba in Canada and from one location in Japan (Table 1.1, Chapter 1).

An unidentified species of *Exophiala* was isolated from the roots of nine *Salix* plants at 49°N, and from one plant at 56°N. This species appeared to increase in abundance at lower latitudes, with higher frequency in rangeland than boreal forest habitats. This *Exophiala* species often inhabited roots where other DSE fungi were absent (Table 2.2), suggesting that it has different habitat preferences than other DSE fungi, its presence excludes other DSE fungi, or the presence of other DSE fungi excludes this species. The species of *Exophiala* isolated here does not correspond to any known species of *Exophiala*, although it is morphologically similar to *E. salmonis* (however, *E. salmonis* possesses 1-septate conidia that do not remain attached to conidiogenous cells). *Exophiala* species belong to the "black yeasts", fungi capable of both filamentous and yeast-like growth that are allied to the Herpotrichiellaceae

(Dothideales) (Untereiner & Naveau 1999); here, one strain of the unidentified *Exophiala* forms a weakly supported clade with *E. salmonis*, *E. pisciphila* and *Fonsecaea pedrosoi* in parsimony analysis of ribosomal DNA sequences (Figure 2.16), suggesting that it is, indeed, a member of the Herpotrichiellaceae.

Exophiala and *Fonsecaea* are common environmental anamorphic fungi associated with soil (e.g. Okeke & Gugnani 1986, Widden 1986, Gocmen & Ozkan 2002); *Exophiala* is also found in decaying wood (e.g. Dixon *et al.* 1980) and water (Nishimura *et al.* 1987). *Exophiala* and *Fonsecaea* species are also agents of disease in humans and other vertebrates (e.g. Pedersen & Langvad 1989, de Hoog *et al.* 2000, Boisseau-Garsaud *et al.* 2002). Some species of *Exophiala* may also act as endophytes: *Exophiala jeanselmei* (Lang.) McGinnis & Padhye was isolated from the rhizosphere of *Cornus canadensis* (Summerbell 1989), and an unidentified species of this genus has been reported as an endophyte of *Abies alba* needles (Sieber-Canavesi & Sieber 1993). *Exophiala pisciphila* McGinnis and Ajello occupies roots as a pathogen of root-inhabiting nematodes (Morgan-Jones & Rodriguez-Kabana 1981), but neither nematodes nor cysts were observed in *Salix* roots from which *Exophiala* was isolated here. It is more likely that *Exophiala* acts as a root endophyte of *Salix* at Latitude One (49°N).

Trimmatostroma species are anamorphs of "loculoascomycetes" (Goodwin & Zismann 2001), a group of often pathogenic ascomycetes that form asci within stromatic locules. Species of *Trimmatostroma* have been recorded as causal agents of leaf spot (Goh & Yipp 1996, Crous & Palm 1999, Taylor & Crous 2000), corticolous fungi (Barengo *et al.* 2000), inhabitants of rotting wood (Farr *et al.* 1989), lichenicolous fungi (Nordin 1996), lithophilic fungi (Sterflinger 1998) and inhabitants of hypersaline environments (Zalar *et al.* 1999). In Canada, *Trimmatostroma* has been isolated from *Populus* and from dead twigs of *Salix* (Conners 1967). Although *T. salicis* and other species of *Trimmatostroma* are endophytes outside the root of *Salix*, this is the first record of a member of this genus found within living roots of *Salix*.

In phylogenetic analysis of ITS2 sequences (Figure 2.15), a sterile isolate (K1) from site K at 78°N formed a weakly supported clade with *Phialophora gregata*

(Allington and Chamberlain) Gams (Figure 2.15), the causal agent of brown stem rot in soybean, adzuki bean and mung bean (Kobayashi *et al.* 1991). This isolate remained sterile in culture on a variety of media (MEA, CMA, MMN, GBA and MLA), a common characteristic of many species of *Phialophora* (W. Untereiner, pers. comm.). Schadt *et al.* (2001) isolated a sterile DSE fungus closely related to *P. gregata* (based on SSU and ITS2 sequence data) in the roots of an alpine plant, *Ranunculus adoneus*. In this study the unnamed fungus (DSE DS16b) of Schadt *et al*. (2001) falls within the *L. orchidicola* clade and not with *P. gregata* (Figure 2.15); this fungus is likely not conspecific to the isolate K1. We cannot conclude that isolate K1 is *P. gregata*. However, it is likely a member of the Leotiales since *P. gregata* is placed in this order (Schadt *et al.* 2001) and this isolate aligns readily here with other Leotialean fungi in analysis of ITS2 sequence data.

Species of the genus *Cadophora* are distinguished from *Phialophora* in having pale to hyaline phialides and / or collarettes. They are anamorphs of the Dermateaceae (Leotiales) (Gams 2000). The isolate identified as *Cadophora* (C1) could not be identified to species because of cultural contamination; however, a species of *Cadophora* [*C. malorum* (Kidd & Beaum.) Gams] is known from plant roots as a pathogen (Blok & Bollen 1995). Here, *Cadophora* was isolated from apparently healthy roots and may not be acting as a pathogen.

Other DSE fungi, including *Chloridium paucisporum*, *Phialophora finlandia* and *Phialocephala dimorphospora* (see Chapter 1) were not isolated from *Salix* roots along our latitudinal transect. *Phialophora finlandia* and *Chloridium paucisporum* are reported rarely in literature, because they rarely sporulate and are therefore isolated but not identified, they have a limited distribution or they are rare associates of plants. *Phialocephala dimorphospora* has been isolated from a variety of substrates in Canada, but not from *Salix* roots.

Two hyaline hyphomycetes were frequently isolated from roots from all latitudes. These were present in most Petri plates and were difficult to separate from the dark, septate fungi. Although hyaline hyphae were not observed within roots, the root squashes were not stained so hyaline root cells could easily mask such hyphae. It is difficult to determine whether these fungi were endophytic, found on the root surface, or were a cultural contaminant, but the frequency of isolation and the lack of other fungal contaminants suggest they were, in fact, also root endophytic.

Up to six taxa of DSE fungi were isolated from root sections of approximately 1cm in length (Figure 2.14). Species of root endophytic fungi co-exist in extremely small areas of root tissue. Jumpponen (1999) and Grünig *et al.* (2001) found that multiple genotypes of *Phialocephala fortinii* inhabit the same root segment (<1 cm in length). It appears that DSE fungi co-exist in relatively small areas, although it remains to be investigated whether spatial partitioning of the roots or resources within roots occurs.

2.4.2 Distribution of DSE fungi along a latitudinal transect in Canada

Dark septate root endophytes have been directly observed in the roots of plants from many locations in Canada, including Alberta (e.g. Currah & Van Dyke 1986, Thormann *et al.* 1999), and Nunavut (Bledsoe *et al.* 1990). Direct observation of roots sampled along our latitudinal transect of Canada revealed DSE structures (dark, septate hyphae and/or microsclerotia) within most roots, with the exception of six from 49°N (Table 2.2). Isolation of DSE fungi from *Salix* roots increased with increasing latitude: DSE fungi were isolated from the roots of 18 of 24 plants sampled at 49°N, 22 of 24 from 56°N, and 23 of 24 plants from both 62°N and 78°N. Further, at 49°N, there is a greater incidence of two DSE species isolated from the same root segment than that at higher latitudes (Figure 2.14). Different recovery rates of DSE fungi at different latitudes may be due to culture bias, temperature and moisture differences at each site, soil characteristics, variations in the composition of plant and fungal communities, or a combination of these and unknown factors. A similar pattern of displacement was noted by Hambleton and Currah (1997) who found the isolation frequency of *P. fortinii* in ericaceous plants decreased in soils with low pH; in these habitats there was an increase in the number of *Oidiodendron maius* isolates relative to *P. fortinii*.

Six root bits from sites RE and RW at 49°N displayed no DSE structures, and ectomycorrhizal structures were completely absent from all root bits observed at this latitude (Table 2.2). Site RE was a slough dominated primarily by *Salix*; and although DSE have been previously isolated from wetlands (e.g. Thormann *et al.* 2001) it is possible that this particular habitat is inhospitable to DSE fungi. Site RW was part of a range actively grazed by cattle. Although DSE have been observed in the roots of plants in rangelands in the United States (e.g. Barrow & Aaltonen 2001), they have not been reported in *Salix* at lower latitudes, apart from at high altitudes (e.g. Jumpponen 1999).

The high frequency of observation and isolation of DSE fungi from *Salix* roots sampled at 56°N and from 62°N was expected since DSE are known from northern boreal forest habitats in Canada (Summerbell 1989, Thormann *et al.* 1999) and from plants that are found in the boreal forest (e.g. Hambleton *et al.* 1998). Although their role in boreal forests is uncertain (see Jumpponen & Trappe 1998), DSE fungi, and *Phialocephala fortinii* in particular, appear to be common root endophytes throughout this habitat.

DSE fungi were isolated from 23 of 24 *Salix* roots collected at 78°N, and DSE structures were observed in root fragments from all plants sampled at this latitude. 78°N represents a high arctic oasis on Ellesmere Island. In a prior survey of root endophytic fungi of high arctic habitats, Kohn and Stasovski (1990) observed little evidence of DSE in the roots of a wide variety of plants collected on Ellesmere Island, including *Salix*, based on detecting dematiaceous hyphae in root squashes. Bledsoe *et al.* (1990) did report DSE-like structures in the roots of plants in the Truelove lowlands on Devon Island in the Canadian arctic. The reason for this discrepancy is unclear. DSE fungi are indeed present in high arctic habitats, although their frequency and broad-scale distribution in these habitats and the nature of their association with arctic plants remains uninvestigated.

DSE fungi occupy a large variety of habitat types in Canada, but not all species are common to all habitat types. Because many dark, septate fungi isolated from roots remain unidentified (e.g. Haselwandter & Read 1982, Summerbell 1989, Ahlich & Sieber 1996) and studies that do identify DSE fungi are often limited in geographical scale (e.g. Summerbell 1989, Ursic & Peterson 1997, Addy *et al.* 2000), it is difficult to determine the natural distributions of individual species of DSE fungi in Canada. Here, DSE fungi were isolated at all four points sampled on a north-south transect of Canada. At the lowest latitude (49°N), a shortgrass prairie, *Exophiala* sp. was most commonly isolated, whereas *Phialocephala fortinii* was most frequently isolated species at higher latitudes (boreal forest and high arctic oasis). Other species of DSE, although present, were rarely isolated at all latitudes. It remains to be investigated whether these results reflect ease of isolation, relative abundances of these fungi in *Salix* roots, or relative abundances in these habitats in general.

2.5 Literature cited

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Table 2.1. Sequences accessioned in GenBank included in phylogenetic analysis of ITS2 sequences to aid in identification of sterile or unidentified strains isolated from Salix roots.

Species	GenBank accession #
Leotialean taxa	
Hymenoscyphus ericae (Read) Korf & Kernan	AY112921
Leptodontidium orchidicola Sigler & Carmichael	AF214577
Phialocephala compacta Kowalski & Kehr	AF486125
Phialocephala dimorphospora Kendrick	AF081434
Phialocephala fortinii Wang & Wilcox	AF214580 AF214581 AF214586 AY046964 AY078128 AY078129 AY078134 AY078136 AY078137 AY078140 AY078143
Phialocephala scopiformis Kowalski & Kehr	AF486126
Phialophora finlandia Wang & Wilcox	AF486119
Phialophora gregata (Allington & Chamberlain) Gams	AF132804
unnamed dark septate root endophyte (DSE) (Schadt *et al.* 2001)	AF168783
Herpotrichiellacean taxa	
Capronia sp.	AF050240

Species	GenBank accession #
Capronia acutiseta Samuels	AF050241
Capronia coronata Samuels	AF050242
Capronia dactylotricha Untereiner *et al.*	AF050243
Capronia epimyces Barr	AF050244 AF050245
Capronia fungicola (Samuels & Müller)	AF050246
Capronia mansonii (Schol-Schwarz) Müller *et al.*	AF050247
Capronia munkii Untereiner	AF050248 AF050249 AF050250
Capronia nigerrima (Bloxam) Barr	AF050251
Capronia parasitica (Ellis & Everhart) Müller *et al.*	AF050252 AF050253
Capronia pilosella (Karsten) Müller *et al.*	AF050254 AF050255
Capronia pulcherrima	AF050256
Capronia semiimersa (Candousseau & Sulmont) Untereiner & Naveau	AF050259 AF050260
Capronia villosa Samuels	AF050261
Cladophialophora carionii Trejos	AF050262
Cladosporium sp.	AF050264 AF050265
Exophiala dermatitiis (Kano) de Hoog	AF050268 AF050269 AF050270

Species	GenBank accession #
Exophiala jeanselmei (Langer.) McGinnis & Padhye	AF050271
Exophiala pisciphila McGinnis & Ajello	AF050272 AF050273
Exophiala salmonis Carmichael	AF050274
Fonsecaea pedrosoi (Carrión) Carrión	AF050275
Phialophora americana (Nannf.) Hughes	AF020279 AF050280
Phialophora verrucosa Medlar	AF050281 AF050282 AF050283
Ramichloridium anceps (Sacc. & Ellis) de Hoog	AF050284 AF050285
Ramichloridium mackenzei Campbell & Al-Hedaithy	AF050288
Rhinocladiella atrovirens Nannf.	AF050289

Table 2.2. Structures observed in root bits adjacent to bits used for isolation, and taxa isolated in pure culture from four sites along a latitudinal transect in Canada.

			Fungal taxa[2]									
Latitude	Site	Structures[1]	PF	LO	EXO	CAD	PHI	TRI	SD1	SRD	UY	LM
49 °N	C1	H				X			X		X	X
49 °N	C2	-			X					X	X	X
49 °N	C3	H, S	X		X							
49 °N	C4	H, S										
49 °N	C5	-										
49 °N	C6	NO								X		
49 °N	C7	H			X					X		X
49 °N	C8	H										X
49 °N	RE1	NO			X						X	X
49 °N	RE2	H			X							X
49 °N	RE3	NO			X							
49 °N	RE4	NO							X			X
49 °N	RE5	NO			X						X	X
49 °N	RE6	H									X	X
49 °N	RE7	H										X
49 °N	RE8	H			X							X
49 °N	RW1	H						X	X			X
49 °N	RW2	NO			X							
49 °N	RW3	H									X	X
49 °N	RW4	H							X		X	X
49 °N	RW5	H							X			X
49 °N	RW6	H										
49 °N	RW7	H										

			Fungal taxa[2]									
Latitude	Site	Structures[1]	PF	LO	EXO	CAD	PHI	TRI	SD1	SRD	UY	LM
49 °N	RW8	H	X									
56 °N	FM1	H		X								
56 °N	FM2	H		X								
56 °N	FM3	H, S, E	X									
56 °N	FM4	-										
56 °N	FM5	H										
56 °N	FM6	H, E	X									X
56 °N	FM7	H, S, E	X									X
56 °N	FM8	H	X									
56 °N	B1	H, S, E	X								X	
56 °N	B2	H, S, E	X									
56 °N	B3	H, S	X									
56 °N	B4	H, S, E	X	X								X
56 °N	B5	H, S, E	X									
56 °N	B6	H, S, E		X								
56 °N	B7	H, S, E	X								X	X
56 °N	B8	H, S, E	X									
56 °N	SP1	H, S, E										X
56 °N	SP2	H, S, E			X						X	
56 °N	SP3	H	X								X	X
56 °N	SP4	H		X							X	
56 °N	SP5	H		X								
56 °N	SP6	H, E	X									
56 °N	SP7	H	X									
56 °N	SP8	H	X									
62 °N	N1	H	X									

			Fungal taxa[2]									
Latitude	Site	Structures[1]	PF	LO	EXO	CAD	PHI	TRI	SD1	SRD	UY	LM
62 °N	N2	H, S	X									
62 °N	N3	H, S	X									
62 °N	N4	H, S	X								X	X
62 °N	N5	H, S										
62 °N	N6	H, S, E	X								X	
62 °N	N7	H, S	X									
62 °N	N8	H	X								X	X
62 °N	W1	-	X									X
62 °N	W2	H	X								X	X
62 °N	W3	H, E	X									
62 °N	W4	-	X									
62 °N	W5	H, S, E	X								X	X
62 °N	W6	H, S	X									X
62 °N	W7	H, S, E	X									X
62 °N	W8	H	X									X
62 °N	T1	H, S	X									
62 °N	T2	H	X									X
62 °N	T3	H, S, E	X									X
62 °N	T4	-	X									
62 °N	T5	H	X									
62 °N	T6	H, E	X									
62 °N	T7	-	X									X
62 °N	T8	H, S	X									X
78 °N	M1	H, S	X									
78 °N	M2	H, E	X									
78 °N	M3	H	X									X

			Fungal taxa[2]									
Latitude	Site	Structures[1]	PF	LO	EXO	CAD	PHI	TRI	SD1	SRD	UY	LM
78 °N	M4	H, S	X									
78 °N	M5	H, S	X									
78 °N	M6	H, S, E	X									
78 °N	M7	-										
78 °N	M8	H, E	X									X
78 °N	D1	H, E	X									
78 °N	D2	H, S	X									
78 °N	D3	H, E	X									
78 °N	D4	H, S, E	X									X
78 °N	D5	H	X									X
78 °N	D6	H, S	X									
78 °N	D7	H, S, E	X									X
78 °N	D8	H	X									
78 °N	K1	H, E					X				X	
78 °N	K2	H	X									X
78 °N	K3	H, E	X									
78 °N	K4	H, S, E										
78 °N	K5	H, E	X	X							X	
78 °N	K6	-	X								X	
78 °N	K7	H, E	X									
78 °N	K8	H, E	X								X	X

[1] Structures: dark septate hyphae (H), microsclerotia (S) or ectomycorrhizal structures (E); "NO" indicates that none of these structures were present, and "-" that the root squash was destroyed during processing.

[2] Fungal taxa: PF = *Phialocephala fortinii*; LO = *Leptodontidium orchidicola*; EXO = *Exophiala* sp.; CAD = *Cadphora* sp.; PHI = *Phialophora* sp.; TRI = *Trimmatostroma* sp.; PHO = *Phoma* sp.; SD1 = sterile DSE 1; SRD = sterile red-staining DSE; UY = unidentified yellow-staining endophyte; LM = *Lecanicillium muscarium*.

Table 2.3. Five cultural types (see text) displayed by *Phialocephala fortinii* strains isolated from *Salix* roots, and the latitudes from which they were isolated.

Latitude	Cultural type 1	2	3	4	5
One (49°N)					X
Two (56°N)	X	X	X		X
Three (62°N)	X	X	X	X	X
Four (78°N)	X	X	X	X	

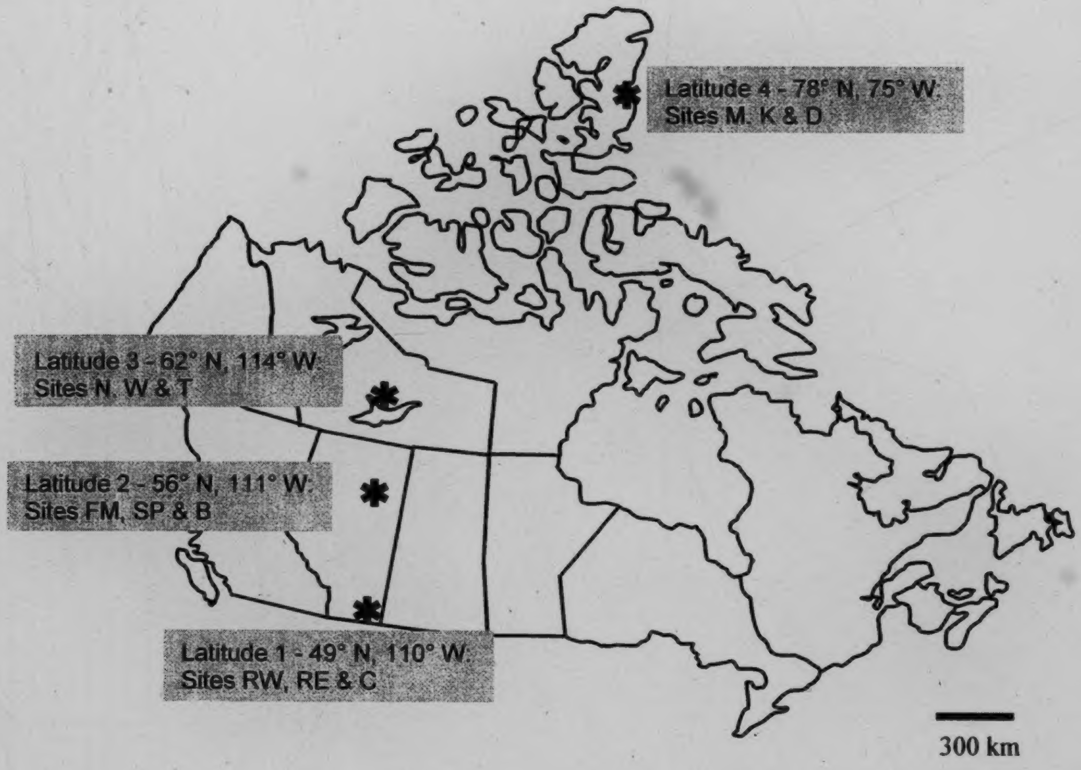

Latitude 4 - 78° N, 75° W:
Sites M, K & D
Latitude 3 - 62° N, 114° W:
Sites N, W & T
Latitude 2 - 56° N, 111° W:
Sites FM, SP & B
Latitude 1 - 49° N, 110° W:
Sites RW, RE & C
300 km

Figure 2.1. Map of Canada showing four latitudes sampled: One Four, Alberta, 49°N; Fort McMurray, Alberta, 56°N; Yellowknife, Northwest Territories, 62°N; and Alexandra Fjord, Nunavut, 49°N. At each latitude, three sites were sampled for the presence of dark septate root endophytic fungi in the roots of *Salix* plants.

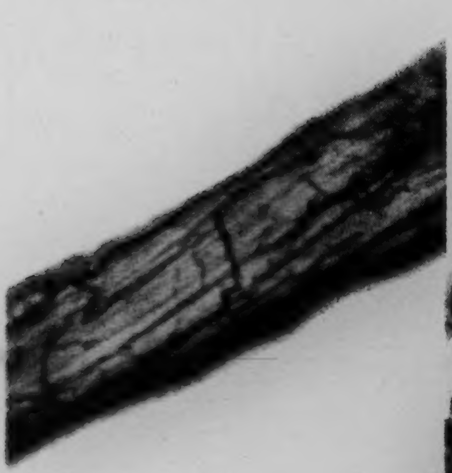
Figures 2.2 to 2.5. Structures observed in *Salix* root segments adjacent to root tissue from which fungi were isolated, typical of structures observed in all roots.

Figure 2.2. Dark septate hyphae among root cells of plant D6 (from site D at 78°N). Scale bar = 10 μm.

Figure 2.3. Intracellular microsclerotia in root of plant D3 (from site D at 78°N). Scale bar = 20 μm.

Figure 2.4. Sparse darkly pigmented mantle tissue (arrow) on root of plant B4 (from site B at 56°N). Scale bar = 20 μm.

Figure 2.5. Dense black mantle tissue on short root tip of plant D3 (from 78°N). Scale bar = 100 μm.



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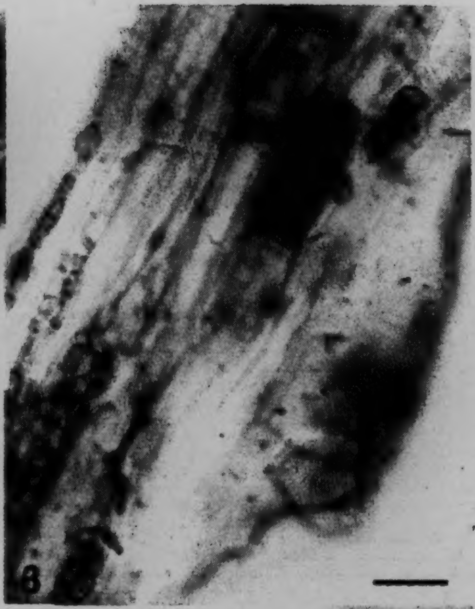

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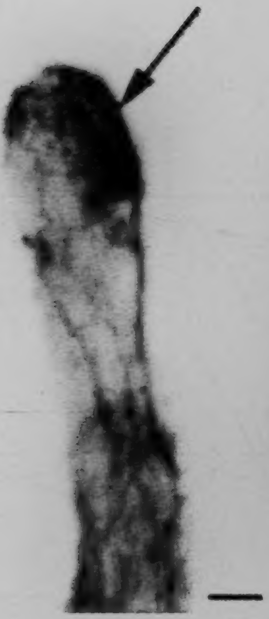

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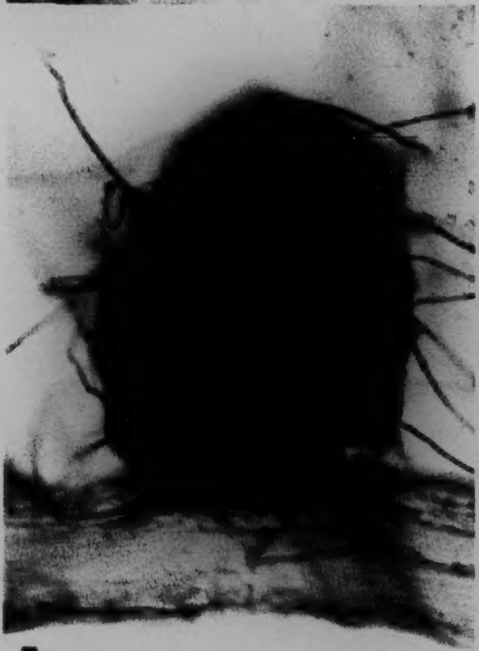

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Figure 2.6. Conidiogenous apparatus of a strain of *Phialopcephala fortinii* isolated from *Salix* roots in site B at 56 °N, and asperate hypha. Scale bar = 15 µm.

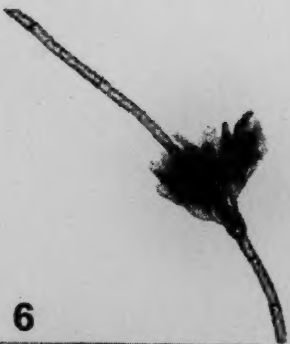
Figure 2.7. Short, peg-like conidiogenous cell produced by *Exophiala* sp. strain RE1 (from site RE at 49°N). Scale bar = 10 µm.

Figure 2.8. Conidiogenous cell with inflated tip produced by *Exophiala* sp. strain C2 (from site C at 49°N). Scale bar = 15 µm.

Figure 2.9. Conidiophore of *Cadophora* sp. isolated from *Salix* roots at 49°N. Scale bar = 10 µm.

Figure 2.10. Stromatic structure produced by *Trimmatostroma* sp. isolated from *Salix* roots in site RW at 49°N. Scale bar = 10 µm.

Figure 2.11. Septate, smooth-walled conidium produced by *Trimmatostroma* sp. isolated from *Salix* roots in site RW at 49°N. Scale bar = 10 µm.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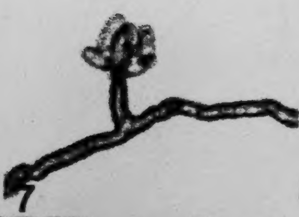
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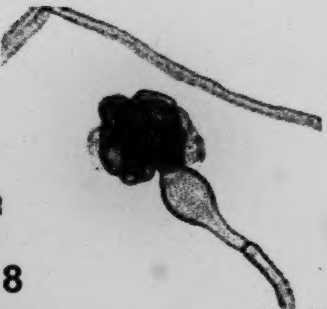
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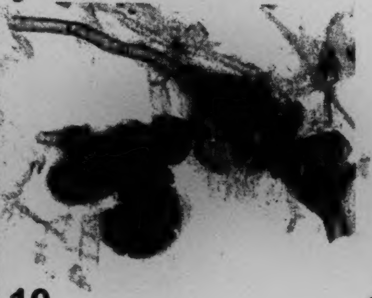

10

11

Figure 2.12. Colony morphology of "sterile DSE 1" on malt extract agar.

Figure 2.13. Colony morphology of "sterile red-staining DSE" on corn meal agar.

12

13

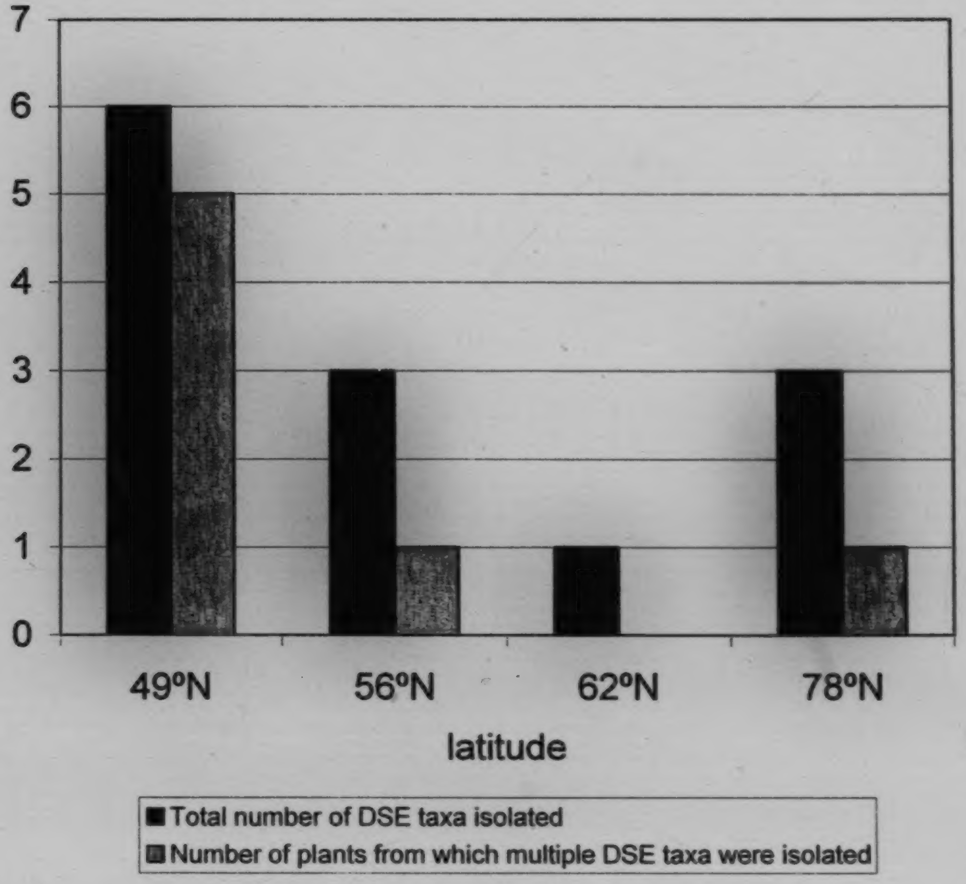


Figure 2.14. Total number of DSE taxa isolated per latitude, and number of root segments per latitude from which two or more DSE taxa were isolated.

Figure 2.15. One of 10 000 most parsimonious trees produced by analysis of sequence data for the second internal transcribed spacer (ITS2) region of the ribosomal DNA from fungi isolated from *Salix* roots along a latitudinal transect in Canada, and sequences accessioned in GenBank (see Table 3.2). Strains that have sporulated in culture are indicated by an asterix. Bootstrap values ≥50% (100 replicates) are given above branches after omitting all but three *Phialocephala fortinii* strains (strains D3 and N6 from this study, and AF214583 from GenBank) from the analysis. A "fast" bootstrap value, using the entire data set, for the *P. fortinii* clade is given below the branch. Arrows indicate branches that collapse in a strict consensus of all 10 000 trees. Tree length = 141 steps, CI = 0.731, RI = 0.657.

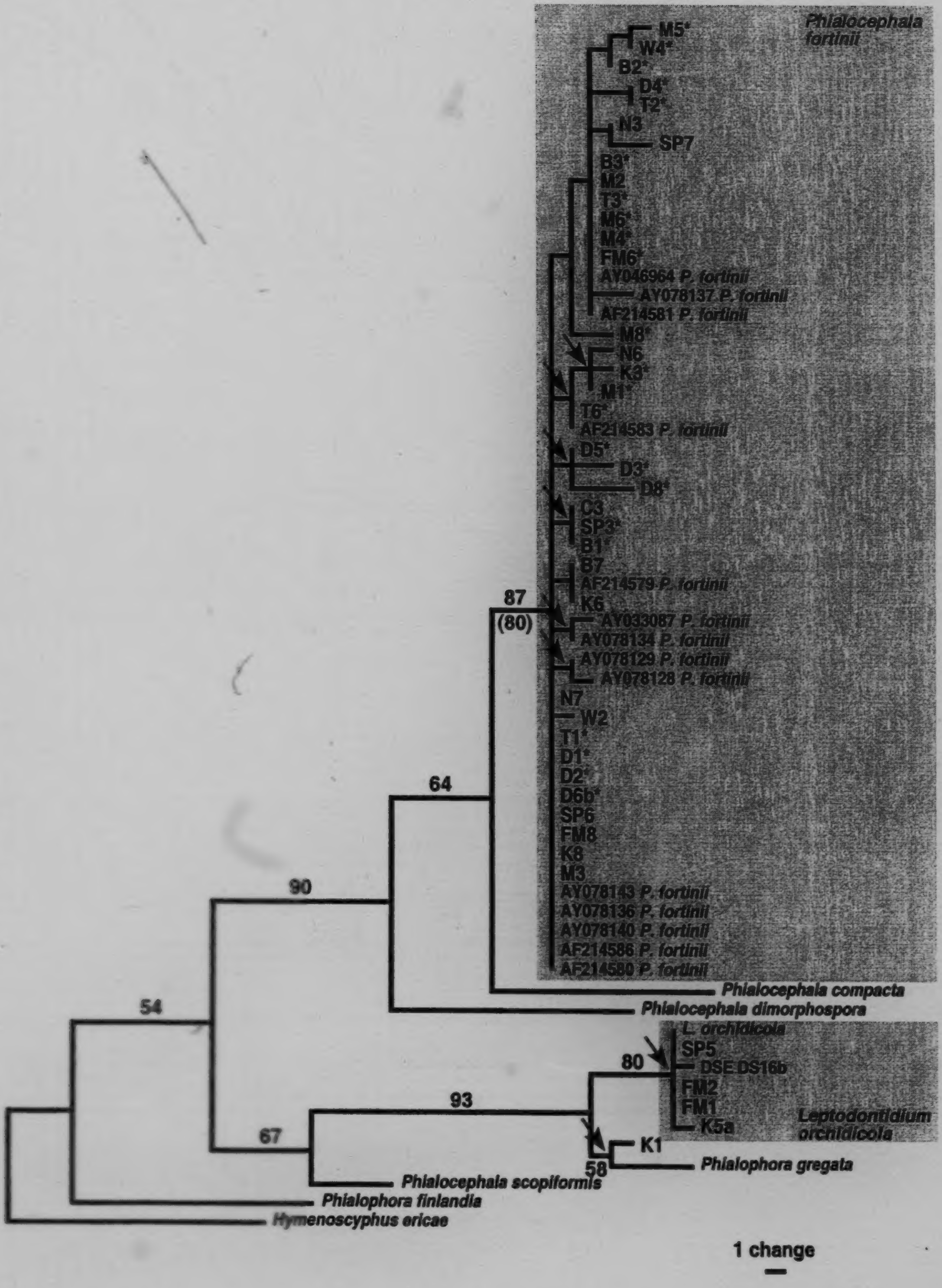
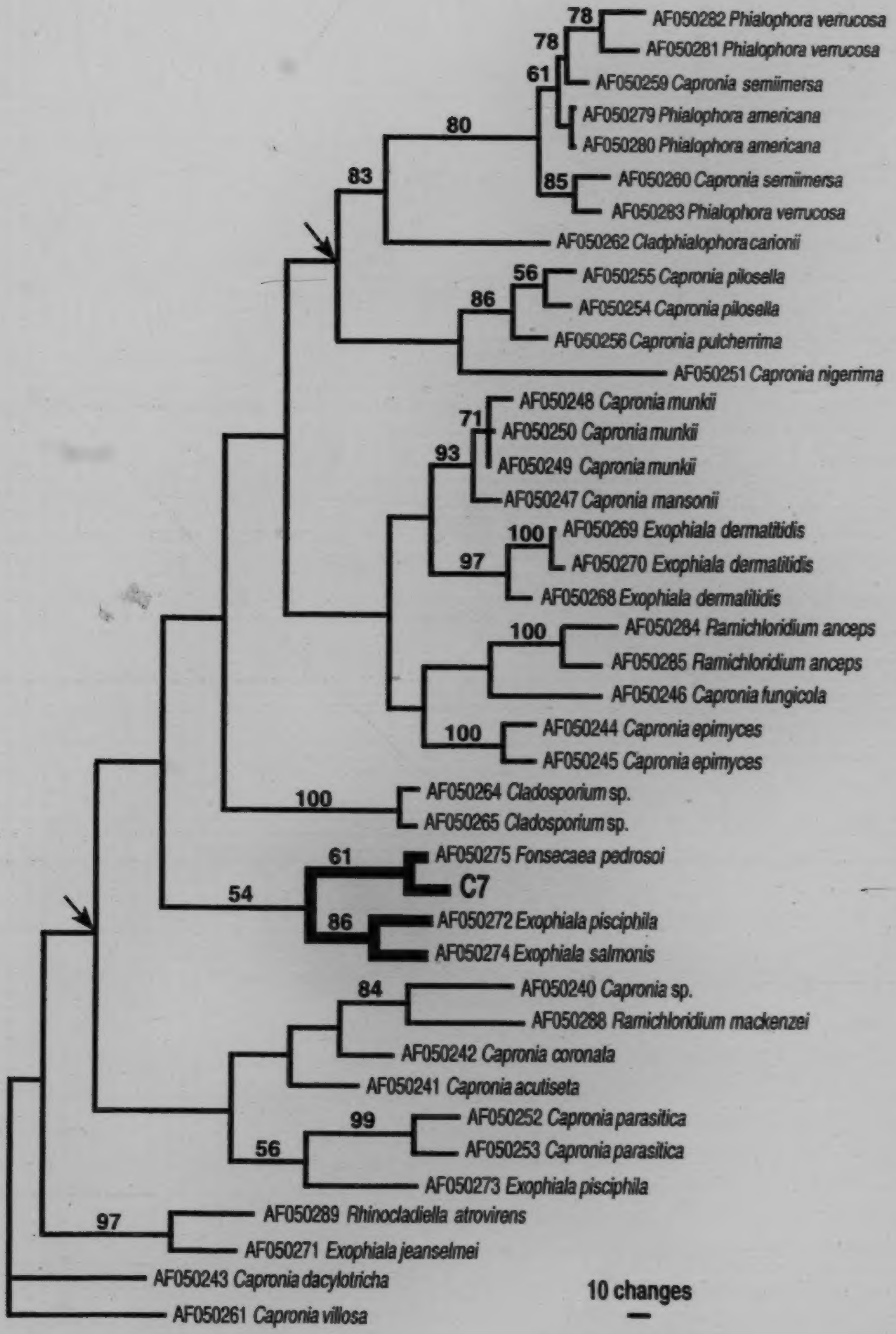

Phialocephala fortinii
M5*
W4*
B2*
D4*
T2*
N3
SP7
B3*
M2
T3*
M6*
M4*
FM6*
AY046964 P. fortinii
AY078137 P. fortinii
AF214581 P. fortinii
M8*
N6
K3*
M1*
T6*
AF214583 P. fortinii
D5*
D3*
D8*
C3
SP3*
B1*
B7
AF214579 P. fortinii
K6
87
(80)
AY033087 P. fortinii
AY078134 P. fortinii
AY078129 P. fortinii
AY078128 P. fortinii
N7
W2
T1*
D1*
D2*
D6b*
SP6
FM8
K8
M3
AY078143 P. fortinii
AY078136 P. fortinii
AY078140 P. fortinii
AF214586 P. fortinii
AF214580 P. fortinii
64
90
54
Phialocephala compacta
Phialocephala dimorphospora
L. orchidicola
SP5
80
DSE DS16b
FM2
FM1
K5a
Leptodontidium orchidicola
93
K1
58
Phialophora gregata
67
Phialocephala scopiformis
Phialophora finlandia
Hymenoscyphus ericae
1 change

Figure 2.16. One of nine most parsimonious trees produced by analysis of sequence data for the second internal transcribed spacer (ITS2) region of the ribosomal DNA from isolate C7 (from site C at 49°N), isolated from *Salix* roots along a latitudinal transect in Canada and identified morphologically as a species of *Exophiala*, and sequences of members of the Herpotrichiellaceae deposited in GenBank (see Table 3.2). Bootstrap values ≥50% (100 replicates) are given above branches. Arrows indicate branches that collapse in a strict consensus of all nine trees. Tree length = 2308 steps, CI = 0.365, RI = 0.480.


78
78
61
80
85
83
AF050282 Phialophora verrucosa
AF050281 Phialophora verrucosa
AF050259 Capronia semiimersa
AF050279 Phialophora americana
AF050280 Phialophora americana
AF050260 Capronia semiimersa
AF050283 Phialophora verrucosa
AF050262 Cladphialophora carionii
56
86
AF050255 Capronia pilosella
AF050254 Capronia pilosella
AF050256 Capronia pulcherrima
AF050251 Capronia nigerrima
71
93
AF050248 Capronia munkii
AF050250 Capronia munkii
AF050249 Capronia munkii
AF050247 Capronia mansonii
100
97
AF050269 Exophiala dermatitidis
AF050270 Exophiala dermatitidis
AF050268 Exophiala dermatitidis
100
AF050284 Ramichloridium anceps
AF050285 Ramichloridium anceps
AF050246 Capronia fungicola
100
AF050244 Capronia epimyces
AF050245 Capronia epimyces
100
AF050264 Cladosporium sp.
AF050265 Cladosporium sp.
61
54
86
AF050275 Fonsecaea pedrosoi
C7
AF050272 Exophiala pisciphila
AF050274 Exophiala salmonis
84
AF050240 Capronia sp.
AF050288 Ramichloridium mackenzei
AF050242 Capronia coronata
AF050241 Capronia acutiseta
99
56
AF050252 Capronia parasitica
AF050253 Capronia parasitica
AF050273 Exophiala pisciphila
97
AF050289 Rhinocladiella atrovirens
AF050271 Exophiala jeanselmei
AF050243 Capronia dacylotricha
AF050261 Capronia villosa
10 changes

Chapter 3: Patterns of genetic variation in *Phialocephala fortinii* across a broad latitudinal transect in Canada

3.1 Introduction

3.1.1 *Phialocephala fortinii*

Phialocephala fortinii Wang & Wilcox is a member of the dark septate endophytes or DSE (Haselwandter & Read 1982), a group of fungi found in apparently healthy roots of many plants worldwide, which encompass a broad range of ascomycetous fungi. These taxa generally produce olivaceous to mouse grey mycelium in culture (Jumpponen & Trappe 1998a). They are difficult to identify because they sporulate rarely or sporadically, often only after extended periods of incubation (4-6 months) at cool temperatures. One taxon in this assemblage that is commonly found is *Phialocephala fortinii*, a hyphomycete that has been isolated from the roots of 51 different plant species from 18 families (Table 1.1, Chapter 1). Because isolates of this species rarely sporulate and are similar in culture to others in the DSE, its global distribution is equivocal, although records suggest *P. fortinii* is most common in cool soils that are rich in organic debris (Addy *et al.* 2000).

3.1.2 Variation in *Phialocephala fortinii*

The role that *Phialocephala fortinii* plays as a root endophyte is not certain. However, the high frequency of documented isolation of this species from the roots of plants indicates that the association is not coincidental. Unfortunately, in vitro studies to determine the mycorrhizal potential of *Phialocephala fortinii* have been inconclusive, even though several authors have used a range of plant species, fungal strains and growing conditions to re-synthesize the symbiosis (Jumpponen & Trappe 1998b); interactions among these three variables have yielded results that indicate mutualism, parasitism or no effect on host fitness (e.g. Fernando & Currah 1996, Wilcox & Wang 1987, Stoyke & Currah 1991).

In culture, the colonial morphology of *Phialocephala fortinii* exhibits considerable variation. For example, colonies may have abundant aerial mycelium or little aerial mycelium but abundant submerged mycelium, and numerous sclerotia or none at all, and solid or feathery margins (Stoyke 1991, Addy et al. 2000). These observations suggest that *P. fortinii* either displays great phenotypic plasticity, and / or is genetically quite variable. The latter supposition has been more or less supported by various types of genetic analysis including polymerase chain reaction / restriction fragment length polymorphism (PCR / RFLP: Stoyke et al. 1992), random amplified polymorphic DNA (RAPD: Jumpponen 1999), ribosomal DNA sequence analysis (Addy *et al.* 2000), and inter-imple-sequence-repeat-anchored polymerase chain reaction (ISSR-PCR: Grünig *et al.* 2001). While these attempts noted genetic variation in *P. fortinii*, none were of a scale sufficient to indicate whether patterns of variation follow a geographic distribution. Neither the patterns of genetic variation nor their sources in P. fortinii are understood. Sexual reproduction by *P. fortinii* has not been observed in situ or in vitro. Sequence analysis of ribosomal genes has placed it within the Leotiales (Lobuglio *et al.* 1996, Jumpponen & Trappe 1998a, Hambleton 1998, Grünig *et al.* 2002b). The observations of premature discoid ascocarps in pot culture by Currah *et al.* (1993) and surprisingly high intra-specific genetic variation has led to speculation that *P. fortinii* may produce sexual reproductive structures that have simply been overlooked (Jumpponen 1998, Grünig *et al.* 2001).

3.1.3 Objective

The objectives of this study were to describe the distribution of *Phialocephala fortinii* across an extensive latitudinal gradient in Canada ranging from 49°N to 78°N, and to characterize its genetic variation along the same gradient. Genetic variation was characterized using amplified fragment length polymorphism (AFLP; Vos et al. 1995) analysis; briefly, AFLP analysis involves restriction of total genomic DNA with two enzymes and ligation of short nucleotide sequences (adaptors) to cut ends, followed by two polymerase chain reaction (PCR) amplification steps with "selective" primers that correspond to the adaptor sequences. Because AFLP analysis samples the complete genome (both coding and non-coding regions), it gives access to a very large range of polymorphisms, making it suitable for intra-species studies of genetic variation. AFLP analysis requires little genomic DNA, is highly reproducible in comparison to some other techniques (Jones *et al.* 1997), and no prior knowledge of DNA sequence for the study organism is necessary.

It is hypothesized that *Phialocephala fortinii* will inhabit the roots of plants of the genus *Salix* sampled at each point along the latitudinal gradient, and that geography will have a measurable effect on the structuring of relatedness among individuals in that: (a) Distinct clones will be indigenous to distinct habitats; and (b) Individuals in populations of the same latitude will be more closely related to each other than to those from populations of other latitudes.

3.2 Materials and methods

3.2.1 Isolation and identification of *Phialocephala fortinii*

Three sites, at least 1.5 km apart, were chosen at each of four latitudes (Figure 3.1). Latitude One was approximately 200 km north of One Four, Alberta, at 49°N - 110°W. Three sites (RW, RE and C) were sampled May 20-21, 2000. Sites RW and C at Latitude One were grazed areas of short grass prairie; site C was in a slough dominated by *Salix*. Sites FM, B and SP at Latitude Two, approximately 20 km east of Fort McMurray, Alberta, at 56°N - 111°W, and within the boreal forest region, were sampled July 12-13, 2000. Sites N, W and T at Latitude Three, approximately 30 km west of Yellowknife, Northwest Territories, at 62°N - 114°W, were sampled between July 20 and 23, 2000. Latitude Four at Alexandra Fjord on Ellesmere Island in Nunavut, Canada, 78°N - 75°W, is a "high arctic oasis." This area has a slightly higher average temperature and soil moisture in relation to the surrounding region because of high cliffs on either side of the sites that trap heat, and sites are on a relatively flat plain that continuously receives glacial runoff water during the growing season. Sites M, K and D were sampled at Latitude Four between July 29 and 31, 2000.

At each site, eight *Salix* plants were selected by stratified random design: A 40 m^2 plot was created, divided into four 20 m^2 quadrats, and two *Salix* plants were selected at random within each quadrat, regardless of age, species, or condition of the plant. *Salix* was selected because representative species of this genus are found along the entire latitudinal transect and *Phialocephala fortinii* is known from *Salix* roots (Jumpponen 1999). Climate and plant community data are summarized in Appendices 1 and 2.

Two contiguous root segments (~5 mm) were excised from each *Salix* plant approximately 30 cm from the main stem. Root segments were surface sterilized for 2 min in 1 part commercial (5.25%) hypochlorite bleach : 2 parts d-H_2O, and cut into approximately 1 mm bits. To maximize recovery of target fungi, root bits were plated onto both corn meal agar with oxytetracycline [CMA+t; 15 g corn meal agar (Fisher Scientific B11132), 10 mg oxytetracycline dihydride (Sigma O-5750), 1 L dd-H_2O] and CMA+t with rose bengal disodium salt (33 mg / L; Sigma R-3877), and incubated at 23°C for two months. Fungi forming darkly pigmented colonies of sterile mycelium with septate hyphae were isolated and stored at 4°C for 4 to 15 months on CMA [15 g corn meal agar (Fisher Scientific B11132), 1 L dd-H_2O] in Petri plates (10 cm diam.)

Sporulating strains could be identified as *Phialocephala fortinii* on the basis of conidiophore and conidium morphology (Wang & Wilcox 1985, Currah *et al.* 1993). Sterile and sporulating strains were grown on tap water agar agar [TWA; 15 g Bacto® agar (Difco 214010), 1 L dd-H_2O] and single hyphal tips were used to produce pure colonies on CMA. Pure strains were grown in still liquid cultures of potato dextrose broth [PDB; 15 g potato dextrose broth (Difco 254920), 1 L dd-H_2O] at 14°C for 2 weeks. Mycelia were rinsed in sterile dd-H_2O to remove residual PDB, and then lyophilized. Genomic DNA was extracted as follows: 0.02 g lyophilized fungal tissue was ground in sterile sand with approximately 2 mg polyvinylpolypyrrolidone (PVPP; Sigma P-6755), then added to 750 µl 2X CTAB buffer (Doyle & Doyle 1987) with 0.2 µl ß-mercaptoethanol (Sigma M-7154) and incubated at 65°C for 1 h. Proteins were removed by adding and mixing 750 µl 24 : 1 chloroform : isoamyl alcohol to the CTAB solution, which was then centrifuged at 13 200 rcf for 20 min. The supernatant was removed and DNA was purified using a QIAquick PCR Purification Kit (Qiagen 28104): 3.5 ml Buffer PB was added to DNA, mixed, and run through a purification column; bound DNA was washed with 750 µl Buffer PE and eluted with 30 µl 65 °C UV-sterilized dd-H_2O. DNA was treated with 0.5 ng RNase A and incubated at 37 °C for 30 min and stored at -20 °C.

3.2.2 Phylogenetic analysis of ITS2 sequence data

The second internal transcribed spacer (ITS2) region of ribosomal DNA was amplified using prITS3 and prITS4 (White *et al.* 1990) with a PE GeneAmp 9700 thermal cycler using standard reaction conditions. The thermal profile for the PCR reactions was: 35 cycles of: 1 min at 94°C, 1 min of 55°C and 2 min at 72°C, with an initial denaturation of 94°C for 2 min and a final extension of 72°C for 7 min. PCR products were visualized using 1.5% agarose gel electrophoresis. The ITS2 region was sequenced using the DYEnamic™ ET Terminator cycle sequencing kit (Amersham Pharmacia) with each of the primers used for amplification, with the following cycling parameters: 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; and visualized with an ABI 377 sequencer. Sequencher 3.0 (Gene Codes Corp., Ann Arbor, Michigan, USA) was used to compile contiguous sequences from resulting electrophoregrams. PCR primer sequences were excluded from contigs, and completed contigs were exported from Sequencher as text files.

ITS2 sequences from *Hymenoscyphus ericae*, *Leptodontidium orchidicola*, *Phialocephala compacta*, *Phialocephala dimorphospora*, *Phialocephala fortinii*, *Phialopcephala scopiformis*, *Phialophora finlandia*, *Phialophora gregata* and an unnamed dark septate endophyte (DS16b, Schadt *et al.* 2001) from NCBI GenBank were also included in the analysis (Table 3.1). Sequences were aligned with CLUSTAL W (Thompson *et al.* 1994). Alignments were exported in PHYLIP format (Felsenstein 1995), then imported into Se-Al 1.0 (Rambaut 1998) for minor adjustment following

Graham *et al.* (2000). A final alignment was exported as a NEXUS format and imported into PAUP * v. 4.10b (Swofford 1999).

An heurstic search was performed in PAUP* v. 4.10b with tree-bisection-reconnection (TBR) branch swapping and default settings. A maximum of 10 000 trees was set to limit analysis time. Bootstrap analysis (Felsenstein 1985) was performed on a reduced data set (all *Phialocephala fortinii* sequences were excluded except strains D3 and N6 from this study, and AF214583 from GenBank) with 1000 replicates. A "fast" bootstrap analysis (1000 replicates) was also performed with all taxa included. Trees were rooted with *Hymenoscyphus ericae* (Grünig *et al.* 2002b).

3.2.3 Population genetic diversity: DNA fingerprints from AFLP patterns

Only one strain of *Phialocephala fortinii* isolated from each *Salix* plant was examined with AFLP, with the exception of plant 6 in site 2 at 78°N where two strains (B6a and B6b) were included to test whether a single root segment may host multiple individuals.

AFLP profiles were generated using an Applied Biosystems Small Plant Genome (50 - 500 Mb) AFLP System. Digestion / ligation and PCR reactions were run according to the ABI AFLP™ Plant Mapping Protocol (Applied Biosystems Inc. 2000) with the following exceptions. Selective amplification reaction volumes were cut in half to reduce costs. Selective PCR product (1.2 µl) and GeneScan™ 500 ROX™ Size Standard (0.2 µl) were precipitated at 42°C using a vacuum centrifuge for 30 minutes, re-hydrated in 1.2 µl formamide and heated to 110°C for 5 min before snap-cooling for >5 min, then loaded onto a Gel Company coarse cellulose comb (CAM-48-020). AFLP fragments were visualized with an ABI 377 sequencer.

All possible selective primer combinations (64 total) were tested with DNA from three different strains of *Phialocephala fortinii*. Primer combinations that produced > 30 bands, at least 30% of which were polymorphic, were used to select the four primer combinations used in this study: Eco-TG with Mse-CAA; Eco-TC with Mse-CAC; Eco-TC with Mse-CAT; and Eco-AG with Mse-CAG.

To test reproducibility of AFLP fragments, the profile of one strain (M1) was repeatedly generated to ensure the resulting band pattern was identical on all gels run.

3.2.4 Matrix compilation

Genescan (PE Applied Biosystems) was used to visualize electrophoregrams. DNA fragments were sized by means of an internal size standard (ROX 500) included in each lane. Genotyper v. 3.7 (PE Applied Biosystems) was used to score categories (specific band sizes) as present (1) or absent (0). Categories were only included if they were unambiguously scorable across all individuals. Unambiguous categories were those with scaled peak heights around 100 or greater that were readily distinguishable from background noise for band presence and absence. Occasionally it was unclear whether bands in different individuals were the same size (number of nucleotides); these categories were also completely excluded from the matrix. Individuals whose AFLP profile consisted of consistently low peaks were not included in the analysis (Table 3.2); repeated attempts to generate scorable AFLP profiles for these individuals failed.

3.2.5 Statistical analyses of AFLP data

A neighbour-joining (NJ) tree was created with PAUP* v. 4.10b using Nei-Li genetic distances (Nei & Li 1979). Bootstrap analysis (Felsenstein 1985) using 1000 replicates was used to assess the robustness of individual clusters on the NJ tree. Analysis of molecular variance (AMOVA) was performed using the Arlequin software package (Schneider *et al.* 2000). AMOVA (Excoffer *et al.* 1992) is a hierarchical analysis of variance that separates and tests tiers of genetic variation, for example, within sites, among sites within latitudes, and among latitudes. In addition to partitioning variation, it calculates a statistic, Φ_{ST}, that is analogous to Wright's F_{ST} (Wright 1965). Φ_{ST} describes the correlation of random pairs of haplotypes drawn from lower tiers (latitudes or sites), relative to the correlation of pairs of random haplotypes drawn from higher tiers (for example, the entire latitudinal transect); nesting of tiers is permitted. Φ_{ST} is thus a measure of population subdivision. The "population" here was taken to be an individual latitude or the entire range. Φ_{ST} values can range from 0 to 1; values close to 0 indicate that there is free recombination (and random mating) across the entire population.

Calculation of the index of association (I_A), a measure of multilocus linkage disequilibrium (Brown *et al.* 1980, Maynard Smith *et al.* 1993), was performed with Multilocus 1.2 (Agapow & Burt 2000). I_A measures the degree of association between multiple loci based on the variation of the genetic distance between individuals, where I_A is close to zero for freely recombining populations and significantly higher than zero for those with detectable linkage disequilibrium (due, for example, to partial clonality). The significance of I_A is assessed by comparison with values calculated from 1000 artificially recombined datasets, where the null hypothesis of free recombination is rejected only if the I_A of the observed data set is significantly larger than the distribution of I_A for the randomized data sets (Burt *et al.* 1996). I_A was only determined for all individuals across the latitude transect because sample sizes were small (<20) for individual latitudes; relatively large (>50) sample sizes are required to detect non-random associations between loci (Brown 1975, Zapata & Alvarez 1993), and inferences made from small sample sizes may not be robust (Milgroom 1996).

3.3 Results

3.3.1 Confirmation of identification of *Phialocephala fortinii*

All individuals included in analyses of AFLP data were identified as *P. fortinii* by morphology of conidiogenous structures when produced, or considered conspecific to *P. fortinii* through analysis of ITS2 sequence data (Table 3.3). An heuristic search of ITS2 sequence data yielded the maximum 10 000 most parsimonious trees with length of 141 steps. In all trees, 10 sterile strains included in the study formed a clade with known *P. fortinii* isolated with 87% bootstrap support (Figure 3.2); all lineages in this clade are very closely related. The strains that we were not able to identify as *P. fortinii* morphologically were intermixed with those that could be identified on the basis of morphology. The near-identity of ITS2 sequences for the isolates that could not be confirmed as *P. fortinii* morphologically with those that could, and the intermingling of these two types in a very well supported clade, all lend strong credence to the conspecificity of these strains.

3.3.2 Neighbour-joining analysis of AFLP data

Each of the 48 strains included in the AFLP analysis (Table 3.3) possessed a unique AFLP banding pattern. There was a total of 334 polymorphic bands from four selective primer combinations.

We identified eight clusters from the results of the neighbour-joining analysis (Figure 3.3) with bootstrap support $\geq$75%. Four clusters (1, 2, 3 and 8) were composed of individuals from two or more latitudes; individuals in clusters 1, 2 and 3 were from adjacent latitudes, and individuals in cluster 8 are from both 56°N and 78°N but not 62°N. One cluster (6) was composed of individuals from different sites at the same latitudes. The remaining three clusters (4, 5 and 7) were each composed of individuals from separate sites at 78°N (Figure 3.4).

Strains lying outside of clusters 1-8 included strains from all latitudes. Strains isolated from the same plant (D6a & D6b, 78°N) included in the analysis were well-supported as representing distinct isolates (Figure 3.3).

3.3.3 Statistical analyses of AFLP data

AMOVA analysis of all individuals indicates that the majority (88.81%) of observed genetic variation is attributable to heterogeneity among individuals within the same site and very little (3.47% & 7.71%) heterogeneity among latitudes or among sites within latitudes, respectively (Table 3.4); $\Phi_{ST} = 0.11187$, p-value < 0.00001. At 78°N, 86.20% of the observed genetic variation was attributable to differences between individuals within sites and 13.80% to differences between sites; $\Phi_{ST} = 0.13804$, p-value < 0.00001 (Table 3.5). At 62°N, 96.76% of total genetic variation was attributable to within-site variation and 3.24% to between-site variation; $\Phi_{ST} = 0.03236$, p-value =

0.00489. At 56°N, 99.45% of total genetic variation was attributable to within-site variation and 0.55% to between-site variation; $\Phi_{ST} = 0.00545$, p-value = 0.00196).

There is non-random association between loci ($I_A = 3.576$, $p < 0.001$; Figure 3.5), suggesting the entire range of *P. fortinii* sampled is in linkage disequilibrium.

3.4 Discussion

3.4.1 Pattern of isolation of *Phialocephala fortinii* across a latitudinal transect

Phialocephala fortinii was isolated most frequently from *Salix* roots collected at Alexandra Fjord on Ellesmere Island, the northernmost site from which collections were made (Table 3.3). In contrast, isolates of *P. fortinii* were recovered rarely from roots collected at the southernmost site near One Four, at 49°N. The different recovery rates of *P. fortinii* from these two extremes may be due to culture bias, variations in the composition of fungal communities, temperature and moisture differences at each site, soil characteristics, or a combination of these and unknown factors. There are no known patterns of host plant specificity in *Phialocephala fortinii* (Stoyke *et al*. 1992, Grünig *et al.* 2001) and thus we consider the effects of host species and plant community type to play a minor role in influencing the recovery rates of *P. fortinii* along our gradient.

Although it seems unlikely that culture bias affected isolation frequencies (collection and isolation procedures were essentially identical for roots obtained at all four latitudes), it is possible that heavier colonization of root tissue by other species of endophytic fungi at lower latitudes displaced *P. fortinii*. There was a high rate of recovery of some distinctive but non-DSE taxa at the One Four locality. (These results are presented and discussed in more detail in Chapter 2). A similar pattern of displacement was noted by Hambleton and Currah (1997) who found the isolation frequency of *P. fortinii* in ericaceous plants decreased in soils with low pH; in these habitats there was an increase in the number of *Oidiodendron maius* isolates relative to *P. fortinii*.

3.4.2 Genetic patterns across the latitudinal transect

Definite clones were not observed at any latitude. Neighbour joining analyses of AFLP polymorphisms showed that no two isolates of *Phialocephala fortinii* were identical, even when sampled from the same root (D6a & D6b). The index of association test suggests that there is substantial linkage disequilibrium within the sampled *P. fortinii* population (Figure 3.5). Milgroom & Cortesi (1999) found strong support for linkage disequilibium using I_A in populations of chestnut blight fungus (*Cryphonectria parasitica*) where perithecia were observed, demonstrating that significant linkage disequilibrium may be detected in sexual populations. Since individuals from all latitudes were included because of constraints in sample size, the detection of linkage disequilibrium in *P. fortinii* could also reflect either isolation by distance or strong selection: combinations of alleles that confer an advantage under strong selection may often be observed together; and if members of geographically isolated subpopulations are pooled, linkage disequilibrium may be detected even though there is recombination occurring within these subpopulations.

There is some genetic structuring observed across the sampled range of *Phialocephala fortinii* ($\Phi_{ST} = 0.11187$; Table 3.3). However, individual latitudes did not represent distinct subpopulations, since genetically similar strains of *P. fortinii* ($\geq$75% bootstrap) could be found at collection locations at quite different latitudes (Figures 3.3, 3.4). Four clusters (1,2, 3 and 8) include strains from sites separated by 800 km or more (Figure 3.4). Individuals from a single site (e.g. sites D & K) include representatives of up to three well-supported AFLP clusters. Three of four clusters comprising individuals from multiple latitudes encompass similar genotypes from neighboring latitudes. However, in cluster 8, individuals are from 56 °N and 78 °N but not from 62 °N. I suggest that these groups defined on the basis of AFLP similarity likely exist along the entire latitudinal gradient, but due to sampling bias (21 isolates or less at each latitude) were not detected in the current study.

Clusters 4, 5 and 7 (Figure 3.3) are each made up of individuals from single sites at the Alexandra Fjord locality. These clusters could be interpreted as being specific to their arctic location, although it is not unlikely that they were simply not detected at the more southerly sites, given that fewer strains of *P. fortinii* from lower latitudes were included in the analyses (Table 3.2).

AMOVA analysis within latitudes reveals a pattern of an increasing percentage of genetic variation attributable to differences among sites as latitude increases (0.55%, 3.2% and 13.8%, at latitudes 78°N, 62°N and 56°N, respectively; Table 3.5). In plants (Mooney & Billings 1961, Billing 1974) and invertebrates (Ward *et al.*1994) capable of both asexual and sexual reproduction, there is greater dependence on asexual means of reproduction as latitude and altitude increase, and a subsequent decrease in variation within populations (and increase in variation among populations) with increasing latitude. This is similar to the pattern observed here, although it is possibly confounded by the declining numbers of individuals isolated as latitude decreases.

3.4.3 Significance of findings and future directions

It is well established that *Phialocephala fortinii* displays a large amount of genetic variation within small areas (Stoyke *et al.* 1992, Jumpponen 1998, Grünig *et al.* 2001, Grünig *et al.* 2002a) but individuals in populations at the same latitude were expected still to be more closely related than those from populations of other latitudes. However, two plants growing within a two meter diameter patch could host genotypes more similar to those collected thousands of kilometers away than to each other.

The mechanics of dispersal in *P. fortinii* are speculative, as are the means by which genetic recombination might occur. *Phialocephala fortinii* produces microsclerotia (Currah *et al.* 1993, Yu *et al.* 2001), strands (Currah and Tsuneda 1993) and conidia (Wang & Wilcox 1985, Currah and Tsuneda 1993), all of which presumably could serve to disseminate established genotypes in much the same manner as other predominantly asexual, root inhabiting fungi (e.g. *Cenoccocum geophilum*: Massicotte 1992; Glomales: Warner *et al.* 1987, Friese & Allen 1993, Michelson 1993, Klironomos & Moutoglis 1999). The index of association test suggests that there is substantial linkage disequilibrium within the sampled *P. fortinii* population (Figure 3.5); this species may reproduce at least partly by asexual means. Sexual reproduction has not been observed beyond a single occurrence of abortive apothecia in pot cultures (Currah *et al.* 1993). This observation indicated that *P. fortinii* is the anamorph of an inoperculate discomycete, which is supported by molecular analyses (Lobuglio *et al.* 1996, Gernandt *et al.* 2001). Teleomorph induction in cultures of Leotialean fungi is difficult and reports of anamorph-teleomorph connections in this lineage are relatively uncommon (e.g. Hambleton *et al.* 1999). The absence of a known teleomorph connection and the indication of linkage disequilibrium is not sufficient to conclude that *P. fortinii* reproduces exclusively by asexual means in nature; indeed, no clones were observed in this study, and this value may actually reflect isolation by distance or strong selection for linked traits.

The genetic structure of *Phialocephala fortinii* may be similar to *Hymenoscyphus ericae*, a widely distributed Leotialean ericoid mycorrhizal fungus that has been shown to exhibit such a wide range of cultural and genotypic variants that it is now considered a species complex (Vrålstad *et al.* 2000). If we accept that *P. fortinii* is a species complex, then the presumed the patterns of genetic variation could correlate with the genetic boundaries of a series of cryptic species.

Taxonomic differentiation at this level is common among fungi that form symbiotic associations with the roots of plants. A well-studied example is the plant pathogen *Rhizoctonia solani*. In this species complex, individuals that are geographically isolated may be genetically similar (Matthew *et al*. 1995, Balali *et al.* 1996) and individuals that are in the same geographic location may be genetically dissimilar. The complex is well known for encompassing genetically distinct anastomosis or vegetative compatibility groups (Ogoshi 1987, Jabaji-Hare *et al.* 1990, Vilgalys & Gonzalez 1990). Individuals of the same vegetative compatibility group are able to form viable vegetative unions or anastomoses, while those of differing vegetative compatibility groups are not. To our knowledge, similar studies with isolates of *P. fortinii* have not been conducted but would be profitable. Narisawa *et al.* (2002) have shown that some strains of *P. fortinii* are potentially effective biocontrol agents, and thus an investigation of vegetative compatibility among the putative anastomosis groups of *P. fortinii* would not only provide a better understanding of the genetic structure of this fungus but might also be of some economic importance.

3.5 Literature Cited

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Table 3.1. Sequences accessioned in GenBank included in phylogenetic analysis of ITS2 sequences to aid in identification of sterile strains isolated from *Salix* roots.

Species	GenBank accession #
Hymenoscyphus ericae (Read) Korf & Kernan	AY112921
Leptodontidium orchidicola Sigler & Carmichael	AF214577
Phialocephala compacta Kowalski & Kehr	AF486125
Phialocephala dimorphospora Kendrick	AF081434
Phialocephala fortinii Wang & Wilcox	AF214580 AF214581 AF214586 AY046964 AY078128 AY078129 AY078134 AY078136 AY078137 AY078140 AY078143
Phialocephala scopiformis Kowalski & Kehr	AF486126
Phialophora finlandia Wang & Wilcox	AF486119
Phialophora gregata (Allington & Chamberlain) Gams	AF132804
unnamed dark septate endophyte DS16b (Schadt *et al.* 2001)	AF168783

Table 3.2. Number of *Phialocephala fortinii* strains isolated from *Salix* root samples from four latitudes, with three sites per latitude (D, M and K at 78°N; N, W and T and 62°N; FM, B and SP at 56°N; and C, RE and RW at 49°N), and the number of these strains included in statistical analyses of AFLP data.

Latitude	Number of *Phialocephala fortinii* strains from each latitude (and from individual sites)	Number of *P. fortinii* strains from each latitude (and from individual sites) included in analyses of AFLP data
78°N	21 (D = 9; M = 7; K = 6)	21 (M = 7; D = 9; K = 5)
62°N	23 (N = 7; W = 8; T = 8)	15 (N = 3; W = 4; T = 8)
56°N	15 (FM = 4; B = 7; SP = 4)	11 (FM = 2; B = 6; SP = 3)
49°N	2 (C = 1; RE = 0; RW = 1)	1* (C = 1; RE = 0; RW = 0)

* The strain from 49°N was excluded from AMOVA and Φ-statistic analyses.

Table 3.3. Strains of *Phialocephala fortinii* included in the AFLP analysis and method of identification (by morphology of conidiogenous cells and / or phylogenetic analysis of ITS2 sequence data.)

Latitude	Site	*Phialocephala fortinii* strain #	Conidiogenous cells produced?	Nested with known *P. fortinii* strains in phylogenetic analysis of ITS data*
78°N	1	M1	Y	Y
	1	M2	N	Y
	1	M3	Y	Y
	1	M4	Y	Y
	1	M5	Y	Y
	1	M6	Y	Y
	1	M8	Y	Y
	2	D1	Y	Y
	2	D2	Y	Y
	2	D3	Y	Y
	2	D4	Y	Y
	2	D5	Y	Y
	2	D6a	Y	N/A
	2	D6b	Y	Y
	2	D7	Y	Y
	2	D8	Y	Y
	3	K2	Y	N/A
	3	K3	Y	Y
	3	K5b	Y	Y
	3	K6	N	Y
	3	K8	N	Y
62°N	1	N3	N	Y
	1	N6	N	Y
	1	N7	N	Y
	2	W2	Y	Y
	2	W4	Y	Y
	2	W6	Y	N/A

	2	W8	Y	N/A
	3	T1	Y	N/A
	3	T2	Y	Y
	3	T3	Y	Y
	3	T4	Y	N/A
	3	T5	Y	N/A
	3	T6	Y	Y
	3	T7	Y	N/A
	3	T8	Y	N/A
56°N	1	FM6	Y	Y
	1	FM8	N	Y
	2	B1	Y	Y
	2	B2	Y	Y
	2	B3	Y	Y
	2	B4	Y	N/A
	2	B5	N	Y
	2	B7	N	Y
	3	SP3	Y	Y
	3	SP6	N	Y
	3	SP7	N	Y
49°N	1	C3	N	Y

*** N/A indicates strain was not included in the phylogenetic analysis because it was identified by morphology of conidiogenous cells.**

Table 3.4. Analysis of molecular variance (AMOVA) of haplotypes of 48 *Phialocephala fortinii* isolates from three latitudes and related Φ-statistics.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	Φ_{ST}	p-value
Among latitudes	2	122.34	1.1848	3.47	-	-
Among sites within latitudes	6	253.51	2.6299	7.71	-	-
Within sites	35	1060.0	30.286	88.8	-	-
Total	43	1435.9	34.101	-	0.1119	< 0.00001

Table 3.5. Analysis of molecular variance (AMOVA) of haplotypes of *Phialocephala fortinii* isolates sampled from three sites at each of three latitudes.

	Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	Φ_{ST}	p-value
56°N	Among sites	2	58.967	0.1589	0.55	-	-
	Within sites	7	202.93	28.990	99.45	-	-
	Total	9	261.90	29.149	-	0.0055	0.0020
62°N	Among sites	2	71.196	1.0501	3.24	-	-
	Within sites	11	345.38	31.398	96.76	-	-
	Total	13	416.57	32.448	-	0.0324	0.0049
78°N	Among sites	2	123.35	4.8206	13.80	-	-
	Within sites	17	511.70	30.100	86.20	-	-
	Total	19	635.05	34.921	-	0.1380	< 0.0001

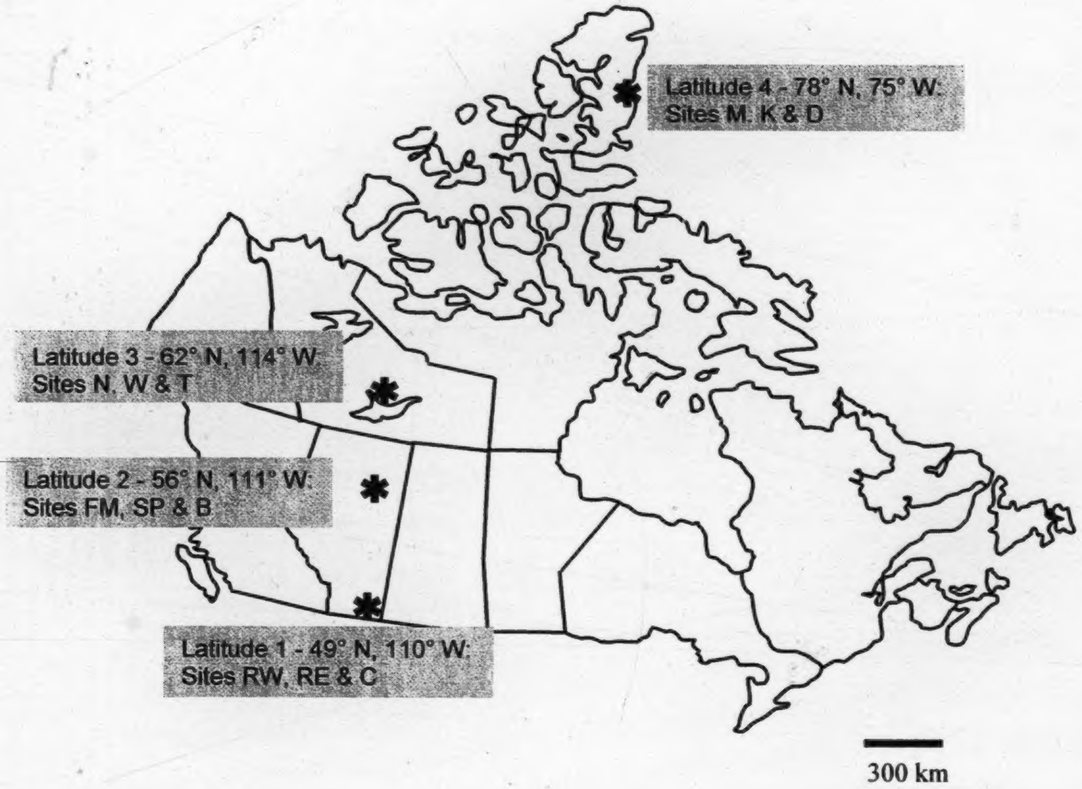

Latitude 4 - 78° N, 75° W:
Sites M, K & D
Latitude 3 - 62° N, 114° W:
Sites N, W & T
Latitude 2 - 56° N, 111° W:
Sites FM, SP & B
Latitude 1 - 49° N, 110° W:
Sites RW, RE & C
300 km

Figure 3.1. Map of Canada showing four latitudes sampled: One Four, Alberta, 49°N; Fort McMurray, Alberta, 56°N; Yellowknife, Northwest Territories, 62°N; and Alexandra Fjord, Nunavut, 49°N. At each latitude, three sites were sampled for the presence of dark septate root endophytic fungi in the roots of *Salix* plants.

Figure 3.2. One of 10 000 most parsimonious trees produced by analysis of sequence data for the second internal transcribed spacer (ITS2) region of the ribosomal DNA from fungi isolated from *Salix* roots along a latitudinal transect in Canada, and sequences accessioned in GenBank (see Table 3.2). Strains that have sporulated in culture are indicated by an asterix. Bootstrap values ≥50% (100 replicates) are given above branches after omitting all but three *P. fortinii* strains from the analysis (see text). A "fast" bootstrap value, using the entire data set, for the *Phialocephala fortinii* clade is given below the branch. Arrows indicate branches that collapse in a strict consensus of all 10 000 trees. Tree length = 141, CI = 0.731, RI = 0.657.

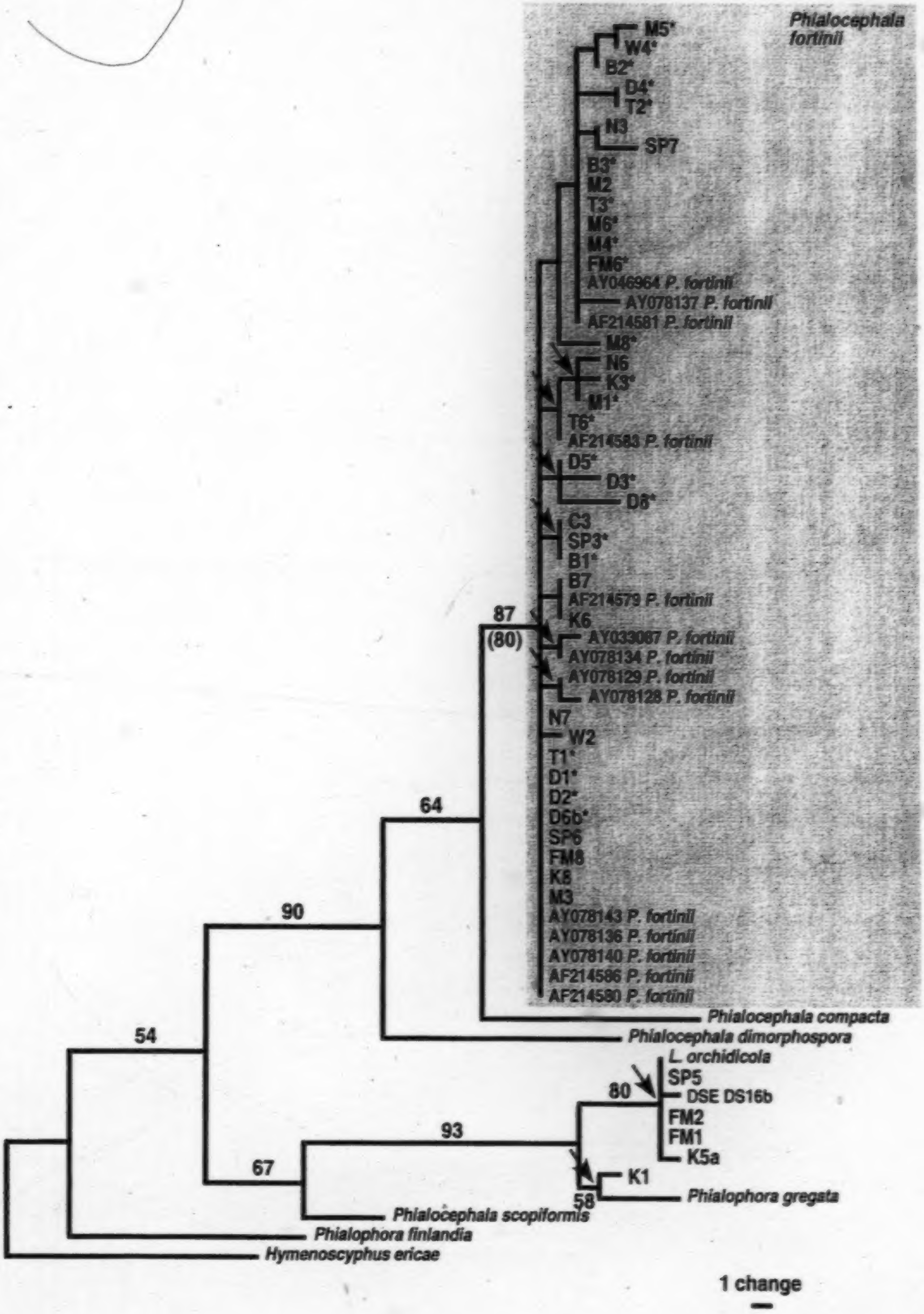
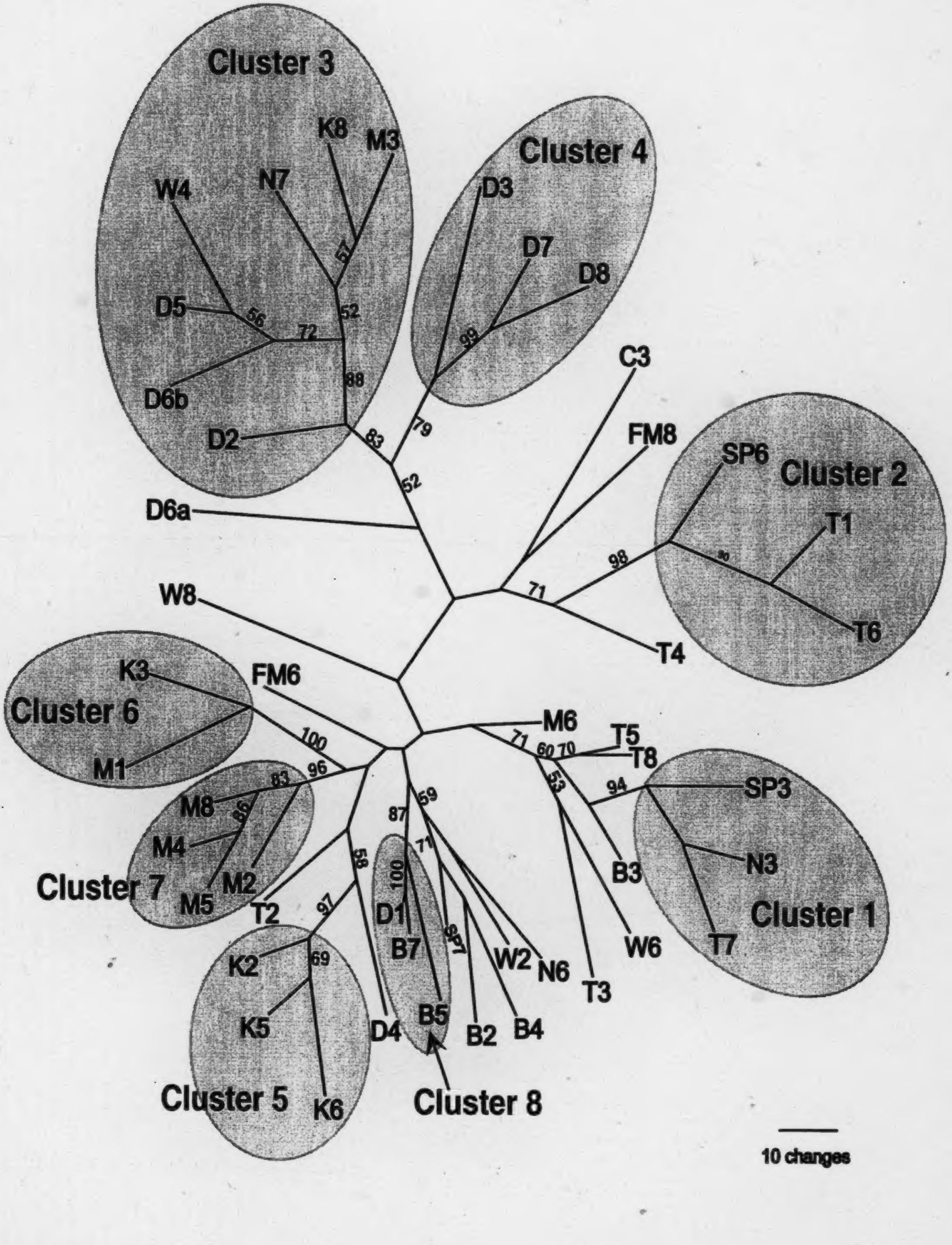

Phialocephala fortinii
M5*
W4*
B2*
D4*
T2*
N3
SP7
B3*
M2
T3*
M6*
M4*
FM6*
AY046964 *P. fortinii*
AY078137 *P. fortinii*
AF214581 *P. fortinii*
M8*
N6
K3*
M1*
T6*
AF214583 *P. fortinii*
D5*
D3*
D8*
C3
SP3*
B1*
B7
AF214579 *P. fortinii*
K6
AY033087 *P. fortinii*
AY078134 *P. fortinii*
AY078129 *P. fortinii*
AY078128 *P. fortinii*
N7
W2
T1*
D1*
D2*
D6b*
SP6
FM8
K8
M3
AY078143 *P. fortinii*
AY078136 *P. fortinii*
AY078140 *P. fortinii*
AF214586 *P. fortinii*
AF214580 *P. fortinii*
87
(80)
64
90
54
Phialocephala compacta
Phialocephala dimorphospora
L. orchidicola
SP5
80
DSE DS16b
FM2
FM1
K5a
93
67
K1
58
Phialophora gregata
Phialocephala scopiformis
Phialophora finlandia
Hymenoscyphus ericae
1 change

Figure 3.3. Unrooted phylogram produced from neighbour-joining analysis of AFLP fingerprints from 48 *Phialocephala fortinii* strains isolated from three sites at each of four latitudes (78°N – sites M, K and D; 62°N – sites N, W and T; 56°N – sites FM, SP and B; and 49°N – site C). Bootstrap values ≥50% (1000 replicates) are given adjacent to nodes; clusters include branches with bootstrap support ≥75%.


Cluster 3
K8
M3
W4
N7
57
D5
56
72
52
88
D6b
D2
83
Cluster 4
D3
D7
D8
99
79
52
D6a
C3
FM8
SP6
Cluster 2
T1
98
71
T6
T4
W8
K3
FM6
Cluster 6
M1
100
96
83
M8
89
M4
M5
M2
Cluster 7
T2
58
97
K2
69
K5
K6
Cluster 5
D4
87
59
100
71
D1
B7
SP7
B5
Cluster 8
B2
B4
W2
N6
M6
71
60
70
T5
T8
53
94
SP3
N3
B3
W6
T3
Cluster 1
T7
10 changes

Latitude	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7	Cluster 8
49°N								
56°N	SP	SP						B
62°N	N, T	T	N					
78°N			M, K, D	D	K	M, K	M	D

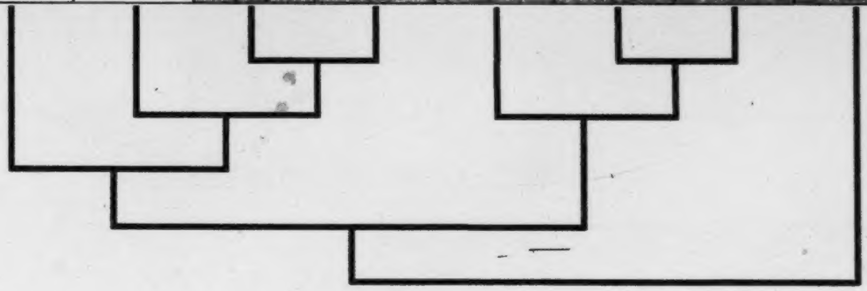


Figure 3.4. Latitudes from which strains of *P. fortinii* were isolated (78°N – sites M, K and D; 62°N – sites N, W and T; 56°N – sites FM, SP and B; and 49°N – site C) and clusters in which they group, based on neighbour-joining analysis of AFLP fingerprint data.

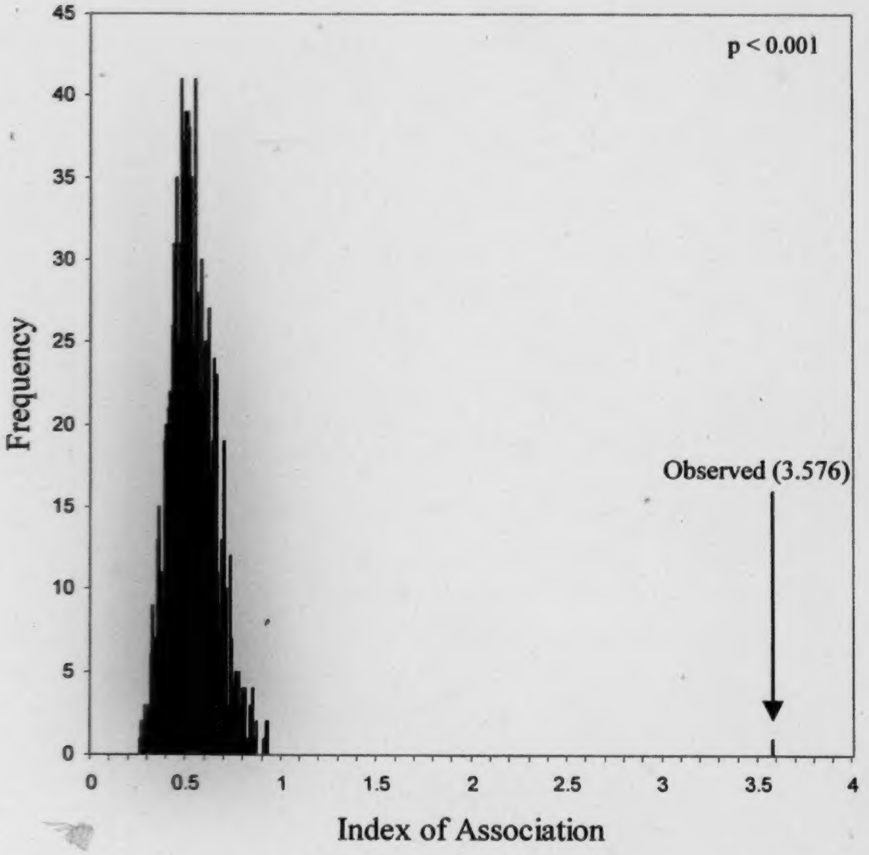


Figure 3.5. Index of association (I_A) value for the observed data set relative to I_A values for 1000 artificially recombined data sets.

Chapter 4. General discussion and conclusions

4.1 Fungi included in the DSE

Dark septate root endophytic (DSE) fungi are ubiquitous in most terrestrial ecosystems (Jumpponen & Trappe 1998a), but little is known about individual species, or even which species to include in this assemblage. The term "DSE" was originally undefined, used loosely to describe dark, septate hyphae observed in roots, dark sterile fungi isolated from roots, comprised of unidentified sterile strains and the five species described by Kendrick (*Phialocephala dimorphospora*), Wang and Wilcox (*Chloridium paucisporum*, *Phialocephala fortinii*, and *Phialophora finlandia*) and Sigler and Currah (*Leptodontidium orchidicola*). Here, DSE fungi are defined more broadly as fungi that have darkly pigmented, septate hyphae and that are frequent or distinctive intracellular associates of roots of apparently healthy plants. Using this definition, a brief literature review yielded four other DSE species: *Hymenoscyphus ericae*, *Oidiodendron maius*, *Heteroconium chaetospira*, and *Phialophora graminicola*. In addition, a species of *Exophiala* was frequently isolated from *Salix* roots from sites at 49°N in this study, and two fungi previously not thought of as belonging to the DSE, *Cadophora* sp. and *Trimmatostroma* sp., were also recorded. To retain the term "DSE" therefore requires an expansion of the species of fungi included in this heterogenous assemblage.

4.1.1 Distribution of DSE fungi in Canada

The current knowledge of the distribution of individual species of DSE fungi is incomplete, due to the fact that many DSE fungi are either observed directly but not isolated from plant roots (e.g. Bledsoe *et al.* 1990), isolated but not identified (e.g. Summerbell 1989), or identified in studies encompassing small geographic areas (e.g. Grünig *et al.* 2002). This is because isolation of DSE fungi from plant roots can be time-consuming, and many DSE fungi remain sterile in culture. Here, DSE fungi were isolated and identified to species in four locations along a latitudinal transect from the Canada-USA border to the high arctic islands of Canada.

DSE fungi are found wherever they are sought in Canada, although the frequency of DSE in roots, and of individual species, changes at different locations. This study represents the first record of *Phialocephala fortinii* from a grassland habitat, but *Phialocephala fortinii* appears to be most abundant in boreal and arctic locales, while in the prairie habitat other species of DSE fungi, such as *Exophiala*, are more frequently isolated. This could be because southern locales are inhospitable to *P. fortinii*, or because *Exophiala* sp. is able to out-compete *P. fortinii* at lower latitudes, or because *P. fortinii* is able to out-compete *Exophiala* sp. at higher latitudes. Although there is insufficient evidence presented here to determine the actual cause of this trend, we may conclude that in different habitats, some species of DSE fungi are more prominent than others.

DSE associations directly observed in plant roots are observed at all latitudes sampled, and appear with greater frequency in the northern boreal forest (62°N) and the high arctic (78°N). DSE are the dominant root endophytes in the Cyperaceae of some alpine communities, and inhabit the roots of over 40 species of plants in Austrian alpine tundra (Read & Haselwandter 1981). Unnamed species of DSE may assist in phosphorus uptake in *Carex* and increase dry weight of its host (Haselwandter & Read 1982). Alpine and arctic communities bear many similarities: they are both cold-dominated, have low availability of soil nutrients, and are often dominated by ericaceous shrubs (Gardes & Dalhberg 1996). It is therefore possible that DSE fungi also play an important role in the high arctic.

4.2 Life history of *Phialocephala fortinii*

The sexual phase of *Phialocephala fortinii* has not been observed. However, there has been much speculation in literature about whether *P. fortinii* is sexual in nature, as it is genetically quite variable (Jumpponen 1999, Grünig *et al.* 2001) and premature apothecia attributable to *P. fortinii* have been observed in pot culture (Currah *et al.* 1993). I am reluctant to draw conclusions on the sexual nature of *P. fortinii* based on the evidence in Chapter 3 of this volume. *Phialocephala fortinii* displays some characteristics of partially asexual species (i.e. populations display linkage disequilibrium); however no clones were isolated in this study.

Some genotypic individuals of *Phialocephala fortinii* were more closely related to individuals from different latitudes than they were to individuals collected from within 10m. Long-range dispersal of *P. fortinii* propagules across the latitudinal gradient is a possible explanation for this pattern, but unfortunately nothing is known about how this fungus actually disperses in nature. To fully comprehend why *P. fortinii* displays the patterns of genetic variation displayed here and in other studies (e.g. Jumpponen 1999, Grünig *et al.* 2001) and the evolutionary history of this enigmatic fungus, the details of its life history must be studied in substantially greater detail.

4.2.1 *Phialocephala fortinii* as a representative member of the DSE

It is important to remember that conclusions drawn about the distribution and population genetic structure of *Phialocephala fortinii* do not necessarily hold true for other species of DSE fungi or for the DSE assemblage in general. *Phialocephala fortinii* is often studied as a representative DSE fungus because it is ubiquitous in many habitats, not host-specific, easily isolated from roots and relatively easy to identify in comparison to many other DSE fungi. However, the role of *P. fortinii* at one location may not hold for *P. fortinii* at another, as it results in different effects on its host plant depending on strain, host plant and / or nutrient conditions (Jumpponen & Trappe 1998b). Further, because DSE fungi display a wide range of life histories, conclusions drawn from study of *P. fortinii* do not necessarily apply to other DSE species; for example, *Phialophora graminicola* displays a distinct sexual phase, *Gaeumannomyces cylindrosporus*, and may have a different population dynamic from a strictly asexual species of DSE fungi.

However, some conclusions common to DSE fungi may still be drawn from study of individual DSE species: DSE fungi are non-specific in their interactions, associate with a wide diversity of hosts, and those tested possess the ability to degrade either organic and inorganic compounds, or both (e.g. Cox *et al.* 1996, Untereiner & Malloch

1999, Caldwell *et al.* 2000, Rice & Currah 2002). The more species of DSE fungi that are identified and studied, the better our understanding of this group will be.

4.3 Future directions

At 49°N, *Exophiala* is the most frequently isolated DSE species, while at all other latitudes sampled *Phialocephala fortinii* was most frequently isolated. It remains to be investigated whether this result reflects relative abundances of these fungi in *Salix* roots, relative abundances in these habitats in general, or antagonistic interactions between *Exophiala* and *Phialocephala fortinii*. In this study and in others, different genetic individuals of the same species as well as individuals of different species are found inhabiting the same root segment. Unfortunately, there are no studies in the literature examining how different DSE species interact with each other. Intraspecies and interspecies competition experiments should be conducted with DSE fungi to better understand how they interact and partition resources.

The *Exophiala* species isolated in this study appears to be unique within this genus. Although the ITS2 segment of the rDNA was sequenced and this species appears to belong to the black yeasts, sequencing the small (18S) and large (28S) subunits of the ribosomal DNA would place this strain with greater confidence within the Herpotrichiellaceae. *Exophiala* species have not been previously thought of as common root endophytes; it would be of great value to determine the ability of the strains isolated here, as well as other species of *Exophiala*, to re-colonize roots of *Salix* and other plants, to determine the morphology of these associations, and investigate the effect *Exophiala* has on its host plant.

Also isolated in this study were three distinct sterile DSE fungi. One (isolate K1) was sequenced for the ITS2 region of rDNA and is suspected to be related to *Phialophora*. It would be valuable to sequence the 5.8S and 28S regions of the rDNA to place these fungi in a broader phylogenetic context. To help identify these fungi to species, further cultural tests should be conducted (e.g. growing these strains on a greater variety of media, and varying environmental parameters such as temperature.)

Knowledge of the details of the life history of *P. fortinii* would help explain the patterns of variation observed in this and other studies. Determining the mode of asexual propagation (e.g. via conidia, microsclerotia, strand, etc.) of *P. fortinii* in nature would aid in determining whether long-range dispersal is possible. There is much speculation in the literature about whether *P. fortinii* is sexual or asexual, but to the best of my knowledge there has been no attempt at pairing different strains under varying growth regimens to induce formation of sexual structures. Vegetative compatibility of genetically distinct *P. fortinii* strains has also not been conducted; the presence of anastomosis groups might help to explain the large observed variation in genotypes at the same field site, and smaller variation among sites (see Chapter 3).

Phialocephala fortinii is commonly thought of as a "representative" DSE fungus. However, it has not been tested whether conclusions drawn from study of *P. fortinii* hold for other DSE fungi. Examination of population genetics of other DSE fungi would be valuable in establishing whether the variation observed in *P. fortinii* is characteristic of other DSE fungi, or an anomaly within the DSE assemblage.

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Appendix 1. Mean climate data from the nearest settlement to each latitude sampled.

	One Four, Alberta, Canada	Fort McMurray, Alberta, Canada	Yellowknife, Northwest Territories, Canada	Alexandra Fjord, Nunavut, Canada
Latitude, longitude	49 °N, 110 °W	56 °N, 111 °W	62 °N, 114 °W	78 °N, 75 °W
Elevation	932 m	369 m	206 m	3-5 m
Dates of sampling	May 20-21, 2000	July 12-13, 2000	July 20-23, 2000	July 29-31, 2000
Mean snow-free days per year [a]	Data not available	206.0	174.4	60-90 [2]
Mean temperature for month of sampling [a]	11.5 °C	16.8 °C	16.8°C	6.2 °C
Annual mean temperature [a]	4.7 °C	0.7 °C	-4.6 °C	-12.3 °C
Annual mean daily low and high temperature [a]	Low: -1.9 °C High: 11.4 °C	Low: -5.3 °C High: 6.7 °C	Low: -9.0 °C High: -0.2 °C	Data not available
Mean precipitation for month of sampling [a]	52.8 mm	81.3 mm	35.0 mm	12 mm [b]
Annual mean precipitation [a]	353.0 mm	455.5 mm	280.7 mm	100-200 mm [b]

[a] Means for One Four, Fort McMurray and Yellowknife based on data collected from 1971 to 2000 as cited by the Meteorological Service of Canada. Means for Alexandra Fjord based on data collected from 1980 to 1988, as cited in Labine (1994).

[b] Values are approximated based on Labine (1994).

Appendix 2. Plant species present at each of three sites from four latitudes along a latitudinal transect in Canada.

Latitude	Site	Woody plant species	Herbaceous plant species
49 °N	C	*Rosa acicularis* *Salix* sp. (or spp.)	unidentified grasses & forbs
49 °N	RE	*Salix* sp. (or spp.)	unidentified grasses & forbs unidentified sedges
49 °N	RW	*Salix* sp. (or spp.)	unidentified grasses & forbs
56 °N	FM	*Cornus canadensis* *Picea glauca* *Populus tremuloides* *Prunus pennsylvanica* *Rhododendron groenlandicum* *Ribes glandulosum* *Salix* sp. (or spp.)	*Achillea millefolium* *Epilobium angustifolium* *Galium* sp. *Maianthemum canadense* *Mertensia paniculata* *Pyrola* sp. *Viburnum edule* unidentified grasses & forbs
56 °N	B	*Cornus canadensis* *Linnaea borealis* *Picea glauca* *Populus balsamifera* *Populus tremuloides* *Prunus pennsylvanica* *Ribes glandulosum* *Salix* sp. (or spp.)	*Achillea millefolium* *Epilobium angustifolium* *Galium* sp. *Maianthemum canadense* *Mertensia paniculata* *Pyrola* sp. *Viburnum edule* unidentified grasses & forbs
56 °N	SP	*Cornus canadensis* *Linnaea borealis* *Picea glauca* *Populus balsamifera* *Populus tremuloides* *Prunus pennsylvanica* *Ribes glandulosum* *Salix* sp. (or spp.)	*Achillea millefolium* *Epilobium angustifolium* *Galium* sp. *Maianthemum canadense* *Mertensia paniculata* *Pyrola* sp. *Viburnum edule* unidentified grasses & forbs
62 °N	N	*Alnus* sp. *Arctostaphylos uva-ursi* *Betula papyrifera* *Linnaea borealis* *Pinus banksiana* *Populus tremuloides* *Prunus* sp. *Rhododendron groenlandicum* *Rosa acicularis* *Salix* sp. (or spp.) *Vaccinium vitis-idaea*	*Lycopodium* sp. unidentified grasses & forbs

62 °N	W	*Alnus* sp. *Arctostaphylos uva-ursi* *Betula payrifera* *Fragaria virginiana* *Picea glauca* *Salix* sp. (or spp.) *Rhododendron groenlandicum* *Vaccinium vitis-idaea*	*Equisetum* spp. *Lycopodium* sp. unidentified grasses & forbs
62 °N	T	*Alnus* sp. *Arctostaphylos uva-ursi* *Linnaea borealis* *Picea glauca* *Pinus banksiana* *Populus tremuloides* *Rhododendron groenlandicum* *Salix* sp. (or spp.) *Vaccinium vitis-idaea*	*Equisetum* spp. unidentified grasses & forbs
78 °N	M	*Cassiope tetragona* *Cerastium alpinum* *Dryas integrifolia* *Oxyria digyna* *Salix arctica*	*Carex* spp. *Papaver lapponicum* *Poa* spp.
78 °N	K	*Cassiope tetragona* *Cerastium alpinum* *Dryas integrifolia* *Oxyria digyna* *Salix arctica*	*Carex* spp. *Papaver lapponicum* *Poa* spp.
78 °N	D	*Cassiope tetragona* *Dryas integrifolia* *Salix arctica* *Vaccinium uliginosum*	*Carex* spp.

Appendix 3. Generalized procedure for detection of amplified fragment length polymorphisms (AFLP), using the Applied Biosystems small plant genome kit.

Step 1:
restriction
-ligation

Genomic DNA is cut using two restriction endonucelases: a rare cutter (*Eco*RI) and a frequent cutter (*Mse*I)

↓

Double-stranded adaptors are ligated to the *Mse*I and the *Eco*RI cut ends of the DNA fragments, creating templates for subsequent PCR amplification steps.

↓

Step 2:
Pre-selective
amplification

First PCR: primers complementary to the adaptors are used to amplify fragments. Primers complementary to the *Mse*I adaptors also possess a 3' C to reduce the number of fragments amplified. Only those genomic fragments that have an adaptor on each end, and have a complementary G to the selective C on the *Mse*I primer, amplify exponentially during PCR amplification.

↓

Step 3:
Selective
amplification

Second PCR: More selective primers are chosen from 16 available AFLP Selective Primers: the *Mse*I primers possess three selective nucleotides, and the *Eco*RI primers possess two selective nucleotides plus a fluorescent dye that can be visualized with a DNA Sequencer on a polyacrylamide gel. Selective amplification with an *Eco*RI and an *Mse*I primer amplifies primarily *Eco*RI-*Mse*I-ended fragments, because the *Eco*RI-*Eco*RI fragments do not amplify well, and the *Mse*I-*Mse*I fragments are not visualized because they do not contain fluorescent dye labels. The result is that only a sub-set of the original restriction fragments are visualized.

Appendix 4. Amplified fragment length polymorphism (AFLP) matrix used in analyses described in Chapter 3, and size (number of nucleotides) of each fragment.

Appendix 4.1. Matrix for selective primer combination *Eco*RI-TG & *Mse*I-CAA.

	103	**104**	**105**	**108**	**109**	**111**	**116**	**117**	**118**	**119**	**127**	**128**	**129**	**132**	**133**	**135**	**138**
B2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B3	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
B4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B5	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
B7	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
C3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
D1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
D2	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
D3	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0
D4	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
D5	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	1
D6a	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
D6b	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
D7	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
D8	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
FM6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FM8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
K2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0
K3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
K5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
K6	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0
K8	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1
M1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
M2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
M3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
M4	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
M5	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
M6	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
M8	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
N3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N6	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
N7	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1
SP3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP6	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
SP7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
T2	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
T3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
T4	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
T5	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
T7	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
T8	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
W2	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
W4	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1
W6	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1
W8	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0

	140	141	143	144	146	150	151	152	153	154	155	157	158	160	161	163	164
B2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B3	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
B4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B7	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
C3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
D1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
D3	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
D4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D5	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
D6a	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
D6b	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
D7	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1
D8	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0
FM6	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
FM8	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
K2	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
K3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
K5	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
K6	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0
K8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
M2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
M3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
M4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M5	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
M6	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
M8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
N6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N7	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
SP3	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0
SP6	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0	0	0
SP7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0
T2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
T3	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0
T4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
T6	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
T7	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0
T8	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
W2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W4	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
W6	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
W8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

	166	167	169	170	172	175	176	178	179	180	182	191	193	194	196	197	198
B2	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
B3	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0
B4	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
B5	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
B7	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
C3	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0
D1	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
D2	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0
D3	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
D4	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0
D5	0	0	0	1	0	1	0	0	0	1	1	1	1	0	0	0	0
D6a	1	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
D6b	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0
D7	0	0	0	1	0	1	0	0	0	0	1	0	0	1	0	0	0
D8	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0
FM6	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0
FM8	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
K2	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0
K3	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0
K5	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	1	0
K6	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0
K8	0	1	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0
M1	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0
M2	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
M3	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0
M4	1	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0
M5	1	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1
M6	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0
M8	1	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	1
N3	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
N6	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
N7	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0
SP3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP6	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
SP7	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
T1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
T2	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	1	0
T3	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
T4	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0	0
T5	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0
T6	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
T7	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
T8	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0
W2	1	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0
W4	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0	0
W6	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0
W8	0	0	0	1	0	1	0	0	1	1	1	0	0	1	0	0	0

	200	201	204	209	211	216	218	220	229	232	239	241	246	248	250	256	258
B2	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
B3	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
B4	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1
B5	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1
B7	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1
C3	1	0	1	1	0	0	1	0	0	0	1	1	1	0	0	0	1
D1	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
D2	1	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0	1
D3	1	0	0	1	0	0	0	0	0	0	1	1	0	1	0	0	0
D4	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
D5	0	1	1	1	0	0	0	0	0	0	1	0	0	1	0	0	0
D6a	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
D6b	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
D7	1	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	0
D8	1	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0
FM6	1	0	0	1	0	0	0	0	1	0	1	1	0	0	1	0	0
FM8	1	0	0	1	0	0	0	1	0	1	1	1	0	0	0	0	0
K2	1	0	0	1	0	0	0	0	0	0	1	1	0	0	1	0	0
K3	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
K5	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
K6	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
K8	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
M1	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
M2	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1
M3	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
M4	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
M5	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
M6	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
M8	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
N3	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
N6	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
N7	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0
SP3	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
SP6	1	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0
SP7	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
T1	1	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0
T2	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
T3	1	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0
T4	1	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0
T5	1	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0
T6	1	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0
T7	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
T8	1	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0
W2	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
W4	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0
W6	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	1
W8	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1

	273	278	279	281	283	284	285	286	290	293	296	298	301	302	307	309	310
B2	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
B3	1	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1
B4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
B5	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
B7	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1
C3	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1
D1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1
D2	1	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	1
D3	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0
D4	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1
D5	0	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0
D6a	1	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	1
D6b	0	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0
D7	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
D8	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0
FM6	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1
FM8	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	1
K2	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1
K3	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1
K5	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1
K6	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1
K8	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0
M1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1
M2	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
M3	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0
M4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M6	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1
M8	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
N3	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1
N6	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
N7	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0
SP3	1	0	0	0	0	0	1	0	1	0	0	0	0	1	1	0	1
SP6	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0
SP7	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
T1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
T2	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1
T3	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
T4	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1
T5	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1
T6	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
T7	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1
T8	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1
W2	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1
W4	0	0	0	0	1	0	0	0	1	0	0	1	1	0	1	0	0
W6	0	1	0	0	1	0	0	0	1	0	0	1	0	0	0	0	1
W8	1	0	0	0	0	0	1	0	1	1	0	0	0	1	0	0	1

	313	314	315	316	320	322	329	334	339	345	352	358	368	375	379	380	386
B2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1
B3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
B4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
B5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
B7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C3	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
D1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
D2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
D3	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1
D4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
D5	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
D6a	0	1	1	1	0	0	0	0	0	0	0	0	1	0	1	0	1
D6b	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
D7	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
D8	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
FM6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
FM8	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
K2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
K3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
K5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
K6	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
K8	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0
M1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1
M2	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1
M3	1	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0
M4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
M5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
M6	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1
M8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
N3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
N6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
N7	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0
SP3	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1
SP6	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1
SP7	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1
T1	0	1	0	0	1	0	0	0	0	1	1	0	1	0	0	0	1
T2	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1
T3	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1
T4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
T5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
T6	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1
T7	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1
T8	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
W2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
W4	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0
W6	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1
W8	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1

	392	395	396	397	400	416	420	430	437	440	447	448	454	457	458	459	461
B2	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0
B3	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
B4	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
B5	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0
B7	1	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0
C3	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
D1	1	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0
D2	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
D3	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
D4	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0
D5	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
D6a	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
D6b	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D7	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
D8	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
FM6	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
FM8	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0
K2	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
K3	1	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0
K5	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0
K6	1	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0
K8	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
M1	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0
M2	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	1
M3	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
M4	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
M5	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
M6	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0
M8	1	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0
N3	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
N6	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
N7	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP3	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
SP6	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
SP7	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0
T1	1	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0
T2	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T3	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
T4	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T5	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T6	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
T7	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T8	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
W2	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0
W4	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
W6	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
W8	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0

	466	467	468	471	475	484	487
B2	1	0	0	0	0	1	0
B3	0	0	0	1	0	0	0
B4	1	0	0	0	0	1	0
B5	0	0	0	0	0	0	1
B7	0	0	0	0	0	0	0
C3	1	0	0	0	0	0	0
D1	0	0	0	0	0	0	0
D2	0	0	0	0	1	0	1
D3	1	0	0	0	0	0	0
D4	1	0	0	0	0	0	0
D5	0	0	0	1	0	0	0
D6a	0	0	0	0	1	0	0
D6b	0	0	0	1	0	0	0
D7	0	0	1	0	0	0	0
D8	0	0	1	0	0	0	0
FM6	1	0	0	0	0	0	0
FM8	0	0	0	0	0	1	0
K2	1	0	0	0	0	0	0
K3	0	0	0	0	0	1	0
K5	1	0	0	0	0	0	0
K6	1	0	0	0	0	0	0
K8	0	0	0	1	0	0	0
M1	0	0	0	0	1	0	0
M2	0	0	0	0	0	0	0
M3	0	0	0	1	0	0	0
M4	0	0	0	0	1	0	0
M5	0	0	0	0	1	0	0
M6	1	0	0	0	0	0	0
M8	0	0	0	0	0	0	1
N3	0	0	0	1	0	1	0
N6	0	0	0	0	0	0	0
N7	0	0	0	1	0	1	0
SP3	1	0	0	0	1	0	0
SP6	0	0	0	0	0	0	0
SP7	0	0	0	0	0	1	0
T1	0	0	0	0	0	0	0
T2	0	0	0	1	0	0	0
T3	0	0	0	0	0	0	0
T4	0	0	0	1	0	0	0
T5	1	0	0	0	0	0	0
T6	0	0	0	0	0	0	0
T7	0	0	0	1	0	0	0
T8	1	0	0	0	0	0	0
W2	0	1	0	0	0	0	0
W4	0	0	0	1	0	1	0
W6	0	1	0	0	0	0	0
W8	0	0	0	0	0	0	1

Appendix 4.2. Matrix for selective primer combination *Eco*RI-TC & *Mse*I-CAC.

	102	109	110	111	125	131	136	138	139	142	146	147	158	163	170	178	188
B2	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
B3	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0
B4	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1
B5	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1
B7	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
C3	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0
D1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0
D2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
D3	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1
D4	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
D5	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
D6a	1	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0
D6b	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
D7	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
D8	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
FM6	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
FM8	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
K2	0	0	0	1	0	1	0	0	1	0	0	1	0	0	1	0	0
K3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
K5	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	0	0
K6	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0
K8	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
M1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1
M2	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1
M3	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1
M4	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1
M5	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1
M6	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
M8	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1
N3	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0
N6	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
N7	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0
SP3	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0
SP6	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0
SP7	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
T1	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0
T2	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0
T3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
T4	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
T6	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0
T7	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
T8	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
W2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
W4	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
W6	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
W8	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1

	189	191	192	194	197	200	202	203	208	209	211	216	217	219	222	223	229
B2	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0
B3	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0
B4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
B5	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
B7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
C3	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
D1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
D2	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
D3	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0
D4	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	0
D5	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0
D6a	0	1	0	0	0	1	1	1	0	0	0	0	0	1	1	0	0
D6b	0	1	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0
D7	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
D8	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
FM6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
FM8	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
K2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
K3	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
K5	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
K6	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
K8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
M1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
M2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
M3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
M4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
M5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
M6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
M8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
N3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
N6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
N7	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
SP3	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0
SP6	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
SP7	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
T1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
T2	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
T3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
T4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
T5	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
T6	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
T7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
T8	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
W2	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0
W4	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0
W6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
W8	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0

	230	232	235	236	241	245	246	250	253	256	257	258	259	267	269	272	280
B2	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0
B3	0	1	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0
B4	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
B5	1	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0
B7	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
C3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
D2	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
D3	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0
D4	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
D5	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
D6a	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0
D6b	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
D7	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
D8	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
FM6	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
FM8	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
K2	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0
K3	1	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0
K5	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
K6	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0
K8	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M1	0	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0
M2	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0
M3	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
M4	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0
M5	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0
M6	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
M8	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
N3	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
N6	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
N7	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
SP3	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
SP6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP7	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
T1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
T2	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
T3	0	1	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0
T4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
T6	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
T7	0	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0
T8	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
W2	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
W4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W6	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
W8	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0

	284	289	291	295	297	311	317	324	326	329	334	39	344	350	356	379	383
B2	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0
B3	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0
B4	0	0	1	0	0	0	0	0	0	1	0	1	0	0	1	0	1
B5	0	0	0	0	0	0	1	1	0	0	1	0	0	0	1	0	1
B7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
C3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
D1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
D2	0	1	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0
D3	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1
D4	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0
D5	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0
D6a	0	1	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0
D6b	0	1	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0
D7	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0
D8	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
FM6	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0
FM8	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
K2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
K3	0	0	0	0	0	1	1	0	0	0	1	0	0	1	1	0	1
K5	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
K6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
K8	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M1	0	0	0	0	0	0	1	0	0	0	1	0	0	1	1	1	1
M2	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
M3	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0
M4	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
M5	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
M6	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
M8	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0
N3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
N6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
N7	0	1	1	0	0	0	1	0	0	1	1	0	1	1	0	0	0
SP3	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0
SP6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
SP7	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0
T1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
T2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
T3	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
T5	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
T6	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
T7	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0
T8	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
W2	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0
W4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W6	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
W8	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0

	388	392	394	396	397	400	406	407	410	416	420	437	443	461	468	483
B2	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0
B3	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
B4	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0
B5	1	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0
B7	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0
C3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D1	1	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0
D2	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
D3	1	0	0	0	1	1	0	0	1	0	1	1	1	1	1	0
D4	1	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0
D5	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1
D6a	1	1	0	1	1	0	0	0	0	0	1	0	1	1	1	1
D6b	1	0	0	0	1	0	0	0	0	0	1	0	0	1	1	1
D7	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
D8	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
FM6	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
FM8	1	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0
K2	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
K3	1	0	0	1	0	0	0	0	1	1	1	0	0	0	1	0
K5	1	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0
K6	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
K8	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
M1	1	1	0	1	0	0	0	1	1	1	1	0	0	0	1	0
M2	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
M3	1	0	0	0	1	1	0	0	0	0	1	0	0	0	1	0
M4	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0
M5	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0
M6	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
M8	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
N3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
N6	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0
N7	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
SP3	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
SP6	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
SP7	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
T1	1	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0
T2	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
T3	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
T4	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
T5	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
T6	1	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0
T7	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
T8	1	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0
W2	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
W4	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1
W6	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
W8	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0

Appendix 4.3. Matrix for selective primer combination *Eco*RI-TC & *Mse*I-CAT.

	101	105	106	110	115	118	125	127	128	130	131	139	141	144	148	154
B2	0	0	1	1	0	1	0	0	1	1	0	1	0	1	0	0
B3	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
B4	0	0	1	1	0	1	0	0	1	1	0	1	0	1	0	0
B5	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
B7	1	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
C[illegible]	0	1	0	0	0	1	0	1	0	0	0	1	1	1	0	0
[illegible]	1	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
D2	0	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0
D3	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0
D4	1	0	0	0	0	1	0	0	1	1	0	1	0	1	0	0
D5	1	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0
D6a	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
D6b	0	0	0	0	1	1	0	1	0	0	0	1	0	1	0	0
D7	0	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0
D8	0	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0
FM6	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
FM8	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0
K2	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
K3	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0
K5	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
K6	0	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0
K8	0	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0
M1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0
M2	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0
M3	0	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0
M4	0	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0
M5	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0
M6	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
M8	0	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0
N3	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
N6	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0
N7	0	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0
SP3	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	1
SP6	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
SP7	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
T1	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
T2	1	0	1	0	0	1	0	0	1	1	0	1	0	1	1	0
T3	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
T4	0	0	1	0	0	1	1	1	0	0	0	1	0	1	0	0
T5	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
T6	0	0	1	0	0	1	1	0	1	0	0	1	0	1	0	0
T7	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
T8	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0
W2	0	0	1	0	0	1	0	0	1	0	1	1	0	1	0	0
W4	1	0	1	0	0	1	0	0	1	0	1	1	0	1	0	0
W6	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0
W8	0	0	0	0	0	1	0	1	0	1	0	1	0	1	0	0

	101	105	106	110	115	118	125	127	128	130	131	139	141	144	148	154
B2	0	0	1	1	0	1	0	0	1	1	0	1	0	1	0	0
B3	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
B4	0	0	1	1	0	1	0	0	1	1	0	1	0	1	0	0
B5	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
B7	1	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
C3	0	1	0	0	0	1	0	1	0	0	0	1	1	1	0	0
D1	1	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
D2	0	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0
D3	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0
D4	1	0	0	0	0	1	0	0	1	1	0	1	0	1	0	0
D5	1	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0
D6a	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
D6b	0	0	0	0	1	1	0	1	0	0	0	1	0	1	0	0
D7	0	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0
D8	0	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0
FM6	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
FM8	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0
K2	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
K3	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0
K5	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
K6	0	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0
K8	0	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0
M1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0
M2	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0
M3	0	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0
M4	0	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0
M5	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0
M6	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
M8	0	0	1	0	0	1	0	0	0	1	1	1	0	1	0	0
N3	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
N6	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0
N7	0	0	1	0	0	1	0	1	0	1	0	1	0	1	0	0
SP3	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	1
SP6	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
SP7	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
T1	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
T2	1	0	1	0	0	1	0	0	1	1	0	1	0	1	1	0
T3	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
T4	0	0	1	0	0	1	1	1	0	0	0	1	0	1	0	0
T5	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0	0
T6	0	0	1	0	0	1	1	0	1	0	0	1	0	1	0	0
T7	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
T8	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0
W2	0	0	1	0	0	1	0	0	1	0	1	1	0	1	0	0
W4	1	0	1	0	0	1	0	0	1	0	1	1	0	1	0	0
W6	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0
W8	0	0	0	0	0	1	0	1	0	1	0	1	0	1	0	0

	160	164	166	169	172	173	174	185	186	192	193	195	197	203	208	212
B2	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
B3	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0
B4	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
B5	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0
B7	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0
C3	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0
D1	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0
D2	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0
D3	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0
D4	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0
D5	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0
D6a	0	1	0	1	0	0	0	0	1	0	0	0	1	1	0	0
D6b	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0
D7	0	1	0	1	0	0	0	0	0	0	0	1	1	0	1	0
D8	0	1	0	1	1	0	0	0	0	0	0	0	1	0	1	1
FM6	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
FM8	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
K2	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0
K3	0	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0
K5	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0
K6	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0
K8	0	0	0	1	0	1	0	0	1	0	0	0	1	1	0	0
M1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1	0
M2	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0
M3	0	0	0	1	0	1	0	0	1	0	0	0	1	1	0	0
M4	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0
M5	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
M6	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0
M8	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
N3	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0
N6	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0
N7	0	0	0	1	0	0	0	1	1	1	0	0	1	1	0	0
SP3	0	1	0	1	0	0	0	1	0	0	0	0	1	1	0	0
SP6	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	1
SP7	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
T1	0	1	1	1	0	0	0	0	0	0	0	0	1	0	1	0
T2	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0
T3	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
T4	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0
T5	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0
T6	0	1	1	1	0	0	0	0	0	0	0	0	1	0	1	0
T7	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
T8	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0
W2	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
W4	0	0	0	1	0	0	0	1	1	0	0	1	0	1	0	0
W6	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0
W8	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0	0

	213	216	220	222	231	233	234	237	242	243	247	249	251	253	254	257
B2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
B3	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
B4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
B5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
B7	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
C3	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
D1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
D2	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1
D3	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	1
D4	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
D5	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1
D6a	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
D6b	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
D7	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1
D8	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
FM6	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
FM8	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
K2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
K3	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
K5	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
K6	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
K8	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1
M1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
M2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
M3	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1
M4	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1
M5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
M6	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
M8	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1
N3	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
N6	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
N7	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0
SP3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
SP6	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
SP7	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
T1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
T2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
T3	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
T6	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
T7	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
T8	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
W2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
W4	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1
W6	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
W8	0	0	0	0	1	0	0	0	0	1	0	1	1	1	0	0

	277	281	283	284	286	289	291	292	295	297	299	301	303	310	315	316
B2	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
B3	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
B4	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
B5	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
B7	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
C3	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0
D1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
D2	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
D3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
D4	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
D5	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
D6a	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
D6b	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
D7	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
D8	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
FM6	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0
FM8	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0
K2	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
K3	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
K5	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0
K6	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0
K8	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
M1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
M2	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
M3	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
M4	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
M5	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
M6	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
M8	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
N3	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
N6	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
N7	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
SP3	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
SP6	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0
SP7	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
T1	0	1	0	0	1	1	0	1	0	1	0	0	0	0	0	0
T2	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
T3	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1
T4	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	1
T5	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
T6	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	1
T7	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
T8	0	0	0	0	1	1	0	0	0	0	0	0	1	0	1	0
W2	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
W4	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
W6	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1
W8	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0

	318	319	321	330	335	338	344	351	357	359	363	367	369	371	372	373
B2	0	0	0	1	0	0	1	1	0	1	0	0	1	0	0	0
B3	0	0	0	1	0	0	1	1	0	0	1	0	1	0	0	1
B4	0	0	0	1	0	0	1	1	0	1	0	0	0	0	0	0
B5	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
B7	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	1
C3	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
D1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	1
D2	0	0	0	1	0	0	1	0	1	0	0	0	0	1	0	0
D3	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0
D4	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
D5	0	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0
D6a	0	1	0	1	0	0	1	1	1	0	0	0	0	0	0	0
D6b	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0
D7	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	1
D8	0	1	0	1	0	0	1	1	1	0	0	0	0	0	0	0
FM6	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
FM8	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0
K2	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
K3	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
K5	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
K6	0	0	0	1	0	0	1	1	0	1	0	0	1	0	0	0
K8	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0
M1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
M2	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
M3	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0	0
M4	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
M5	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1
M6	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	1
M8	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
N3	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1
N6	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0
N7	0	0	0	1	1	0	1	0	1	0	1	0	0	1	0	0
SP3	0	0	0	1	0	0	1	1	0	0	1	0	1	0	0	1
SP6	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0
SP7	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
T1	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0
T2	0	0	0	1	0	0	1	1	0	1	0	1	0	0	0	0
T3	0	0	0	1	0	0	1	1	0	0	1	0	1	0	0	1
T4	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0
T5	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1
T6	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0
T7	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1
T8	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1
W2	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
W4	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0
W6	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0
W8	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0

	377	378	381	383	387	391	399	401	410	416	420	422	423	426	427	429
B2	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
B3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
B4	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
B5	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0
B7	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
C3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
D1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
D2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
D4	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
D5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D6a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D6b	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D7	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
D8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FM6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FM8	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
K2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
K5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
M1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
M2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
M3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
M4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
M5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
M6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M8	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
N3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
N6	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
N7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
SP3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP6	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
SP7	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
T1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W8	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0

	430	439	440	442	444	445	448	449	452	453	454	459	461	464	477	483
B2	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
B3	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0
B4	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1
B5	0	0	0	1	0	1	1	0	0	0	0	0	0	1	0	0
B7	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0
C3	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
D1	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0
D2	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0
D3	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0
D4	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
D5	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
D6a	0	0	0	1	1	0	0	0	0	0	1	0	0	0	1	0
D6b	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
D7	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
D8	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
FM6	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
FM8	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
K2	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
K3	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
K5	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
K6	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
K8	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0
M1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
M2	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
M3	0	0	0	1	0	1	1	0	0	0	1	0	0	0	1	0
M4	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
M5	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
M6	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
M8	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
N3	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0
N6	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
N7	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0
SP3	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0
SP6	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
SP7	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
T1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
T2	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
T3	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0
T4	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
T5	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0
T6	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
T7	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0
T8	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0
W2	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
W4	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
W6	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0
W8	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0

Appendix 3.4. Matrix for selective primer combination *Eco*RI-AG & *Mse*I-CAG.

	100	104	114	116	17	120	123	128	136	138	173	174	175	178	183	191	195
B2	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0
B3	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
B4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
B7	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
C3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
D2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
D3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
D4	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0
D5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
D6a	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
D6b	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
D7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D8	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FM6	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
FM8	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
K2	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
K3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
K5	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
K6	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
K8	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
M1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
M2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
M3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
M4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M6	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1
M8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
N6	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
N7	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0
SP3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP6	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
SP7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T1	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	1	0
T2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T4	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0
T5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
T6	0	0	0	0	1	0	0	0	1	0	1	1	0	0	0	0	0
T7	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
T8	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
W2	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0
W4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
W6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W8	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0

	198	212	215	220	224	225	226	227	230	241	246	248	255	265	281	285	287
B2	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0
B3	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0
B4	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
B5	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0
B7	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
C3	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
D1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
D2	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
D3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D4	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	0	0
D5	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
D6a	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
D6b	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D7	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
D8	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
FM6	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0
FM8	0	1	0	0	0	0	0	1	1	0	0	1	0	0	1	1	0
K2	0	0	1	1	0	0	0	0	0	1	0	0	1	0	1	0	0
K3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
K5	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
K6	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
K8	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0
M1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	0
M2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
M3	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0
M4	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
M5	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
M6	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0
M8	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
N3	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
N6	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0
N7	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0
SP3	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
SP6	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
SP7	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0
T1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
T2	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	0	0
T3	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0
T4	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
T5	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0
T6	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
T7	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0
T8	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0
W2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
W4	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0
W6	0	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	0
W8	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0

	289	291	294	298	307	309	311	316	321	333	339	352	356	360	363	369	389
B2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B5	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
B7	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
D1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D4	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
D5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
D6a	0	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0
D6b	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
D7	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
D8	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
FM6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FM8	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0
K2	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
K3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K5	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
K6	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
K8	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
M2	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
M3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M4	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
M5	0	1	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0
M6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M8	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
N3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N7	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SP7	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
T1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T2	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
T3	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
T4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
T7	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
W6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W8	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

	390	394	396	399	425	427	431	437	443	470	474	491	497
B2	0	0	0	1	0	0	1	0	0	1	1	0	0
B3	0	0	0	1	0	0	0	0	0	1	0	0	0
B4	0	0	0	1	0	0	0	0	0	0	0	0	0
B5	0	0	0	1	0	0	0	0	0	1	0	0	0
B7	0	0	0	1	0	0	0	0	0	1	0	0	0
C3	0	0	0	1	0	0	0	0	0	1	0	0	0
D1	0	0	0	1	0	0	0	0	0	1	0	0	0
D2	0	0	0	0	0	0	1	0	0	1	0	0	0
D3	0	0	0	0	0	0	1	1	0	1	0	0	1
D4	0	0	0	1	0	0	1	0	0	1	0	0	0
D5	0	0	0	0	0	0	1	0	0	1	0	0	0
D6a	0	0	0	1	0	0	1	0	0	0	0	0	0
D6b	0	0	0	0	0	0	1	0	0	0	0	0	0
D7	0	0	0	0	0	0	1	0	0	0	0	0	1
D8	0	0	0	0	0	0	1	0	0	1	0	1	1
FM6	0	0	0	1	0	0	1	0	0	1	0	0	0
FM8	0	0	0	0	0	1	0	0	0	1	0	0	0
K2	0	1	0	1	0	0	1	0	0	1	0	0	0
K3	0	0	0	1	0	0	1	0	0	1	0	0	0
K5	0	1	0	1	0	0	1	0	0	1	0	0	0
K6	0	1	0	1	0	0	1	0	0	1	0	0	0
K8	0	0	0	0	0	0	1	0	0	1	0	0	0
M1	0	0	0	1	0	0	0	0	0	1	0	0	0
M2	0	0	0	1	0	0	1	0	0	1	0	0	0
M3	0	0	0	0	0	0	1	0	0	1	0	0	0
M4	0	0	0	1	0	0	1	0	0	1	0	0	0
M5	0	0	0	1	0	0	1	0	0	1	0	0	0
M6	0	0	0	1	0	0	0	0	0	1	0	0	0
M8	0	0	0	1	0	0	1	0	0	1	0	0	0
N3	0	0	0	1	0	0	0	0	0	1	0	0	0
N6	0	0	0	1	0	0	0	0	0	1	0	0	0
N7	0	0	0	0	0	0	1	0	0	1	0	0	0
SP3	0	0	0	1	1	0	0	0	0	0	0	0	0
SP6	0	0	1	0	0	0	0	0	0	1	0	0	0
SP7	1	0	0	1	0	0	1	0	0	1	1	0	0
T1	0	0	0	0	0	0	1	0	0	1	0	0	0
T2	0	0	0	1	0	0	1	0	0	1	0	0	0
T3	0	0	0	1	0	0	0	0	0	1	0	0	0
T4	0	0	1	0	0	0	1	0	0	1	0	0	0
T5	0	0	0	1	0	0	0	0	0	1	0	0	0
T6	0	0	0	1	0	0	1	0	0	1	0	0	0
T7	0	0	0	1	0	0	0	0	0	1	0	0	0
T8	1	0	0	1	0	0	1	0	0	1	0	0	0
W2	0	1	0	1	0	0	0	0	0	0	0	0	0
W4	0	0	0	0	0	0	1	0	0	1	0	0	0
W6	0	0	0	1	1	0	0	0	0	1	0	0	0
W8	0	0	0	1	0	0	0	0	1	1	0	0	0